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COEXISTENCE OF A THYMOMA AND HODGKIN'S DISEASE OF THE THYMUS

A Case Report

B RIDELL and S LARSSON

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Ridell B & Larsson S Coexistence of a thymoma and Hodgkin s disease of the thymus A case report. Acta path microbiol scand Sect A 88 1–4, 1980

A case of simultaneous occurrence of a thymoma and Hodgkin s disease of the thymus is described

Key words Thymoma Hodgkin s disease of the thymus

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In most instances thymic tumours have a characteristic mode of growth and microscopical picture Some tumours of the thymus however have long been a challenge to pathologists owing to their granulomatous appearance and have been considered variously as a variant of thymoma or Hodgkin s disease of the thymus The present report deals with a thymic mass that contained two distinct tumours one of which was a thymoma and the other Hodgkin s disease of the thymus

CASE HISTORY

A 58 year-old man was admitted to Sahlgrenska sjukhuset in 1972 owing to severe pruritus His general condition was good and no abnormalities were noted at the physical examinations A radiological examination of the chest revealed an abnormal shadow at the level of the arch of the aorta which was considered to be a tumour of mediastinal origin At mediastinoscopy a few enlarged lymph nodes were found under the carina Histologically these lymph nodes were anthracotic and without



Fig 1 Hodgkin s disease of the thymus Tumour nodules surrounded by sclerotic bands (Originally paraffin-embedding Specimen re-embedded in methacrylate May-Grunwald Giemsa × 30)

SUPPLEMENTS

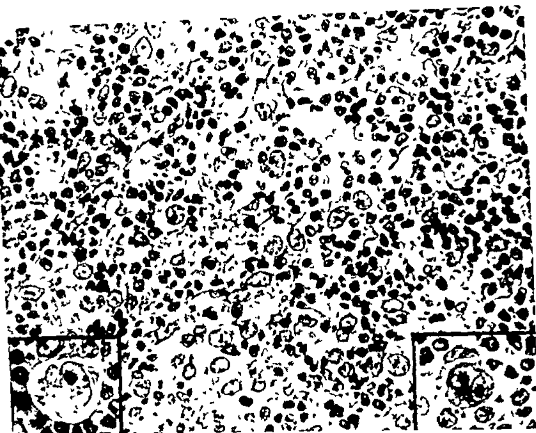


Fig 3 Accumulations of large atypical cells in a mediastinal lymph node (× 460) Lacunar giant cell (left inset) and Reed Sternberg cell (right inset) in the same node (× 720) (Originally paraffin-embedding Specimen re-embedded in methacrylate May Grunwald Giemsa)

tumour and supplementary radiotherapy is an efficacious mode of therapy in Hodgkin s disease of the thymus (*Bergh et al* 1978 b)

It has been stated that a second malignancy is found in 21 per cent of individuals with a thymoma when followed up over a 20 year period (*Soadjian et al* 1968) In our material of thymomas (*Bergh et al* 1978 a) there has not been any other case of a second malignant tumour during a mean observa tion period of 10 years The coexistence of a thymoma and Hodgkin s disease must be considered a pure coincidence

The present case provides an interesting contribu tion to the discussion about granulomatous lesions in the thymic region The simultaneous occurrence of an epithelial thymoma and Hodgkin s disease of the thymus as separate tumours supports the view that Hodgkin s disease of the thymus is a distinct : thymomas

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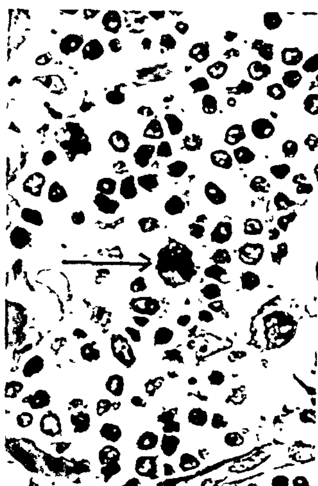


Fig 2 Reed Sternberg cell in thymic tumour (arrow) (Originally paraffin embedding Specimen re embedded in methacrylate May-Grunwald-Giemsa × 720)

signs of tumour Bronchoscopy did not reveal any primary lung tumour At thoracotomy the tumour was located in the mediastinum It partly enclosed the left subclavian and common carotid arteries The tumour reached the posterior surface of the sternum and infiltrated the pericardium and the left anonymous vein and vagal nerve The tumour was removed, together with the remnants of the thymus The anonymous vein the vagal nerve and parts of the pericardium and pleura had to be resected The postoperative course was uneventful Immediately after the operation the patient spontaneously remarked that the pruritus had completely disappeared

The operation specimen weighed 275 g The cut surface of the tumour had solid nodular areas surrounded by thick fibrous bands with a vague demarcation in the periphery Remnants of the upper thymus horns were seen in the upper part of the specimen On the left lateral side of the specimen a separate, well-demarcated soft tumour with a diameter of 4 cm was seen

Microscopically, the large tumour was nodular with the tumour nodules surrounded by sclerotic bands of varying breadth (Fig 1) The nodular areas had a varying cell density with a mixed cellular composition Most of the cells were small lymphocytes but thymic epithelial cells and occasional eosinophils and plasma cells were also present In the nodules there was an irregular distribution of large multinucleated cells, some of which were Reed-Sternberg cells (Fig 2) In the periphery of the tumour remnants of thymic tissue were found In some areas, thymic epithelium was identified in the tumour A lymph node taken at the pulmonary artery contained vaguely nodular tumour tissue with scattered atypical giant cells, some of which were lacunar cells others Reed Sternberg cells (Fig 3) The picture here was considered to be consistent with Hodgkin's disease nodular sclerosis

Three other lymph nodes taken from the vicinity of the trachea were normal The separate, well-demarcated tumour was microscopically an epithelial thymoma (Fig 4)

The patient received full-course radiotherapy postoperatively He is alive and well, free from clinical disease six years after the operation

COMMENT

The present case was found in a retrospective analysis of patients with primary tumours in the thymic region operated upon at Sahlgrenska sjukhuset during the period 1954–1975 (*Bergh et al* 1978 a & b) This series comprised 43 thymomas and 17 cases of Hodgkin's disease of the thymus

A thymic origin of the present tumour seems likely judging by findings of thymic tissue in the periphery of and in the tumour, and the macroscopic findings of two separate tumours with different microscopic patterns were interpreted as evidence that the mediastinal mass was composed of two different tumours

The controversial problem of the classification of some tumours of the thymus as granulomatous thymomas or Hodgkin's disease of the thymus has been debated for many years (*Lowenhaupt* 1961, *Bloodworth* 1975, *Rosai* 1976) However, reviews of these tumours have established that there is often recurrent disease in other parts of the lymphatic system, which microscopically is Hodgkin s disease (*Katz and Lattes* 1969, *Keller and Castleman* 1974) The traditional reluctance to use the term Hodgkin's disease might partly be due to the fact that otherwise characteristic thymomas have been reported to contain granulomatous areas (*Thomson and Thackray* 1957) without sclerosis and that the localized tumours with a granulomatous appearance had a better prognosis than Hodkin s disease usually has Some reports have established, however that the prognosis for Hodgkin s disease limited to one site is good (*Burke et al* 1967) Surgical removal of the



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BASAL CELL CARCINOMA IN THE HEAD AND NECK

The Importance of Location and Histological Picture Studied with a New Scoring System in Predicting Recurrences

LARS-ERIK AFZELIUS ANITA EHNHAGE and HANS NORDGREN

The Departments of Otorhinolaryngology and Pathology University Hospital Lund Sweden

Afzelius L. E. Ehnhage A. & Nordgren H. Basal cell carcinoma in the head and neck The importance of location and histological picture studied by a new scoring system in predicting recurrences Acta path microbiol scand Sect A 88 5– 9 1980

A scoring system for judging the aggressiveness of basal cell carcinomas of the head and neck is devised based upon 258 tumours from 123 patients This system is discussed and we suggest using the depth of growth the degree of palisading and the narrow epithelial strand formation (narrow strands) as a scoring basis It is shown that tumours located on the ear are more aggressive than tumours in other places Tumours on the nose often recur even though their histological picture is not that aggressive This phenomenon is also discussed For proper scoring an excisional biopsy should be performed unless the tumour is very small Recurrent tumours and tumours treated with X ray also have a higher score

Key words Basal cell carcinoma head and neck histological scoring system recurrences

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Received 17 ii 79 Accepted 26 vi 79

During recent years an impression has grown among clinicians as well as among pathologists that some basal cell carcinomas of the head and neck region are more aggressive than most other basal cell carcinomas and that these were usually located in the middle of the face and on the ear These tumours also seem to have a higher rate of recurrence

In the literature these problems have been discussed to some extent *Mora and Robins* (1978) found basal cell carcinoma growing in the triangle of the face (formed by lines drawn from the outer canthi to the philtrum of the lip) tended to be more invasive more destructive more recurrent and more difficult to treat than those in other sites *Bart et al* (1978) noted a higher rate of recurrence in tumours located on the ear and lips (very few cases) periocularly and on the nose and scalp than on other sites *Jackson and Adams* (1973) denied any differences according to the location but their tables indicate a slight tendency towards more horrifying basal cell carcinomas located on the scalp ears lids and canthi and nose compared with other sites

Some reports have touched upon the correlation between prognosis and histology *Thackray* (1951) made up a classification system and found a correlation only with infiltrative growth *Teloh* (1953) postulated that the only two significant factors were the degree of histology inflammatory reaction and the degree of histological invasion *Jackson and Adams* (1973) could not find any correlation between the histological picture and the biological behaviour *Sloane* (1977) also devised a classification system and found a correlation between infiltrative growth and outcome *Dellon* (1978) showed that the more aggressive tumours had a greater differentiation to the squamous epithelium and more irregular palisading than less aggressive

The aim of the present study is firstly to create a histological classification system of basal cell

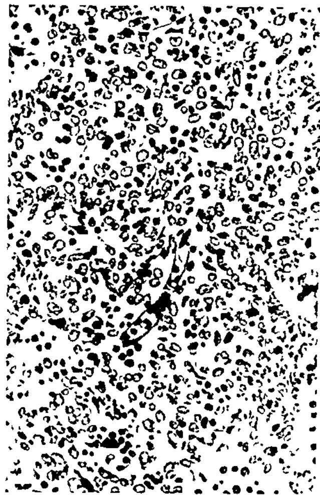


Fig 4. Epithelial thymoma (Originally paraffin-embedding Specimen re-embedded in methacrylate May-Grunwald-Giemsa × 310)

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The Importance of Location and Histological Picture, Studied with a New Scoring System in Predicting Recurrences

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The aim of the present study is firstly to create a histological classification system of basal cell carcinoma and secondly on the basis of this system to discuss the histological variation of the tumours related to location recurrence and X ray treatment thirdly to give some guidelines for the treatment of such tumours

MATERIAL AND METHOD

The material consists of 123 patients treated for basal cell carcinomas of the head and neck during a 6 year period (1971–1976) at the ENT Department University Hospital Lund Sweden Sixty per cent of the patients were males and 40% females The total number of tumours was 258

This is a retrospective study consisting of patients referred to surgery for primary treatment and patients previously treated surgically and/or radiologically with recurrent tumours

Eight of the patients were treated many years ago with radiotherapy to the head and neck region for other reasons mostly tuberculosis The head and neck region was divided into 7 locations *ear* – ear and 1 cm around it *front* – the forehead from the scalp down to and including the eyebrows *eye* – the eyelids and canthi *nose* – the nose and ½ cm around it except the canthi *mouth* – the lips and 1 cm around *cheek* – cheeks with a lower margin at the lower part of the mandible the other margins at the aforementioned locations *miscellaneous* – the rest of the head and neck

All histological examinations were carried out on sections from paraffin embedded formalin fixed tissue stained with hematoxylin and erythrosin

The excised specimens were revised by one of us (HN) and a scoring system was created In this scoring system the depth of the growth (using *Clark s* system (1968) for malignant melanomas) multifocality palisading amount of squamous epithelial differentiation cell differentiation (*Broders* 1941) degrees of fibroplasia of the stroma and growth in narrow epithelial strands (narrow strands) were judged (Table 1 Fig 1 A B) Recurrences were defined as new tumour growth in the old operation site after radical surgical excision or adequate (according to ICRU report 29 (1978)) X ray treatment

For statistical analysis of the difference between the different scores the non parametric Mann Whitney U test was used and the calculations were carried out on a Compucord computer (327)



Fig 1 a Basal cell carcinoma with well defined borders well developed palisading score 1 and slight fibroplasia in the stroma score 1
Haematoxylin and erythrosin × 125

Fig 1 b Basal cell carcinoma infiltrating in narrow epithelial strands showing nearly complete loss of basal cell palisades score 3 Marked fibroplasia in the stroma score 3
Haematoxylin and erythrosin × 125

TABLE 1 *The Different Parameters and Their Respective Scores*

Parameter	Score range*)
Depth of Growth	1 – 5
Loss of Palisading	1 – 3
Fibroplasia	0 – 3
Narrow Strands	0 – 3
Multifocality	0 – 1
Squamos Epithelial Differentiation	0 – 3
Cell Differentiation	1 – 4
Maximum score	22

*) Increasing score with increasing degree of change

RESULTS

The most common location for the basal cell carcinoma was on and around the ear, followed by the nose (Table 2) This table also gives the mean for different parameters in the scoring system and the mean of the total score from the different locations Tumours located in the ear region have a significantly ($p < 0\ 01$) higher score than tumours from the other locations

It is shown in Table 3 that the scoring done on the whole excised tumour is higher than on the biopsy

TABLE 3 *The Mean of Total Score for Biopsy and Whole Excised Tumours in Different Locations*

Location	Biopsy Limited	Excision
Ear	12 0	15 3
Nose	10 5	12 8
Eye	10 9	12 0
Cheek	10 2	10 4
Front	11 8	11 7
Mouth	9 9	11 9
Miscell	10 9	11 2

Thirty two recurrences were noted in the material Most of the recurrences were found in the ear and nose locations and occurred mostly after X ray treatment (Table 4) The mean score for the recurrences *14 6* is *significantly higher* ($p < 0\ 01$) than the mean score for the non recurrence group, 10 8 Even the mean score for the biopsies taken before the recurrences gives a higher but not significant value 11 6 The tumours from the ear and nose locations were further analyzed in order to find parameters in the scoring system of importance in predicting recurrences The depth of growth the narrow strands and the degrees of fibroplasia and loss of palisading were found to be most relevant (Table 5) These 4 parameters have significantly higher scores ($p < 0\ 01$) on the ear in the recurrence group than in the non recurrence group Concerning the nose the differences are not that

TABLE 2 *The Mean Score for the Different Parameters in the Scoring System the Number of Tumours and the Mean of Total Score in the Different Locations*

Parameter \ Location	Ear	Nose	Eye	Cheek	Front	Mouth lip	Miscellaneous	
Depth of growth	3 5	3 6	3 0	3 0	3 2	3 0	3 0	
Loss of palisading	2 4	2 1	2 1	2 1	2 2	1 8	2 0	
Fibroplasia	1 6	1 4	1 3	1 2	1 6	1 2	1 3	
Narrow strand	1 6	1 0	1 2	0 8	1 4	1 0	1 0	
Multifocality	0 8	0 6	0 6	0 7	0 6	0 6	0 7	
Squamous epithelial differentiation	0 9	0 8	1 0	0 5	0 9	0 7	0 9	
Cell differentiation	2 4	2 2	2 2	2 2	1 9	1 9	2 1	
Mean total score	13 2	11 7	11 4	10 6	11 8	10 2	11 0	
Number of tumours	66	65	27	24	16	15	47	M = 11 4

TABLE 4 *Number of Recurrences after X-Ray and Radical Excision in Different Locations*

Location	Number of recurrences after X-ray	Radical excision	Total
Ear	4	3	7
Eye	1	1	2
Cheek	3	1	4
Mouth-lip	1	0	1
Front	0	0	0
Nose	10	1	11
Miscellaneous	5	2	7
Total	24	8	32

convincing, except for narrow strands ($p < 0.01$) In the tumours prior to the recurrences the scores of these parameters were slightly higher than for the non-recurrences, except for the depth of growth in the nose region All except one of these recurrences came after X-ray treatment and diagnoses were made on limited biopsies

It seems as if X-radiation makes the scores higher. In 24 tumours scoring was made before and after X-ray. The mean score before X-ray was 11 1 and after 13 6

TABLE 5 *Total Mean Score for the Different Parameters in the Scoring System for Tumours Prior to the Recurrences (Prior) the Recurrences (Rec) and the Non-Recurrences (Non-Rec) in the Ear and Nose Locations*

Parameter \ Location		Ear	Nose
Depth of growth	Prior	3 9	3 2
	Rec	4 4	3 7
	Non rec	3 6	3 6
Loss of palisading	Prior	2 6	2 2
	Rec	3 0	2 3
	Non rec	2 3	2 1
Fibroplasia	Prior	1 8	1 1
	Rec	2 3	1 4
	Non-rec	1 5	1 0
Narrow strand	Prior	1 7	1 1
	Rec	2 6	1 6
	Non rec	1 4	0 9
Multifocality	Prior	0 9	0 5
	Rec	1 0	0 6
	Non-rec	0 8	0 5
Squamous epithelial differentiation	Prior	0 6	0 7
	Rec	1 0	0 8
	Non rec	0 9	0 7
Cell differentiation	Prior	2 1	2 0
	Rec	2 4	2 1
	Non-rec	2 5	2 0

DISCUSSION

The impetus for this study came from the impression that some basal cell carcinomas of the head and neck region were more aggressive than others and that such tumours were preferably located around the nose and ear We searched for a scoring system to make it possible to judge the aggressiveness of each tumour The literature is rather confusing on this issue and previously no definitive scoring system has been published We, therefore, suggest a system covering all the important parameters in the histological assessment of the basal cell carcinoma

It is important to create a scoring system easy to use by every pathologist As each interpretation of a specimen is somewhat subjective it would be advantageous to have many criteria On the other hand an extensive system can be too impractical for the pathologist and thus not be accepted in the routine work

Our scoring system is constructed of parameters which express the growth pattern of the tumours, such as depth of growth, multifocality, narrow strands, loss of palisading and degree of fibroplasia, and by parameters reflecting the cell appearance, such as the cell differentiation and degree of squamous epithelial maturity The last parameter was chosen in order to detect the so-called metatypical carcinoma (*Selidam et al* 1974)

The material is to some extent selective, in that only a part of it consists of new untreated cases, a great number are recurrences after earlier treatment with surgery and/or X-ray This fact makes it impossible to estimate the frequency of recurrence in this material, consequently no comparisons with other case materials can be made in this report

On the basis of our scoring system it is evident that tumours on and around the ear are the histologically most aggressive In accordance with this there are many recurrences seen on the ear

What is the reason for this? The structure of the tissue in this very special localization? A longer case history? The thickness of the skin on the ear is sparse and practically no subcutaneous fat is present These facts can contribute to a higher Clark score The location as such can increase the patient's delay, and the time factor could be a reason for the more aggressive behaviour of the tumour The

medical records unfortunately often do not record adequate information about the age of the neoplasm

The localization with the greatest problems from a clinical point of view (most often recurrences) is on the nose Tumours located there had however a score not significantly different from the material as a whole The explanation may be technical reasons the margins are often narrower than in other places where a good cosmetic result can be achieved even though ample margin is taken.

The evaluation of the scoring parameters in Table 5 clearly shows that the parameters concerning the cell appearance have no influence on the recurrences This finding is in accordance with *Headington* (1974) The parameters of growth pattern especially depth palisading fibroplasia and narrow strands are the most important factors in the development of a recurrence The last two parameters are well correlated and used by some authors in the morphea concept (*Caro and Howell* 1951) Multifocality thought to be of importance for recurrence (*Sloane* 1977) is a somewhat obscure concept, since it is dependent on the section technique It was not found relevant in this study

To make the scoring system more convenient it could be restricted to the three parameters mentioned above and our proposed scoring system would then be based upon a score with a maximum of 11 (depth – 5 loss of palisading – 3 and narrow strands – 3)

It thus seems that in our material the following conclusions can be drawn. basal cell carcinoma localized on the ear should generally be regarded as potentially more malignant than on other locations in the head and neck region In accordance with this we advise more extensive excision margins like those taken around squamous-cell carcinoma of tumours in this location. Further in this location we advocate excisional biopsies unless the tumour is very small because limited biopsies give less information and a falsely low score Recurrent tumours and tumours treated earlier with X rays should also be treated with wider margins as above

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TABLE 4 *Number of Recurrences after X Ray and Radical Excision in Different Locations*

Location	Number of recurrences after X ray	Radical excision	Total
Ear	4	3	7
Eye	1	1	2
Cheek	3	1	4
Mouth lip	1	0	1
Front	0	0	0
Nose	10	1	11
Miscellaneous	5	2	7
Total	24	8	32

convincing except for narrow strands ($p < 0.01$) In the tumours prior to the recurrences the scores of these parameters were slightly higher than for the non recurrences except for the depth of growth in the nose region All except one of these recurrences

TABLE 5 *Total Mean Score for the Different Parameters in the Scoring System for Tumours Prior to the Recurrences (Prior) the Recurrences (Rec) and the Non Recurrences (Non Rec) in the Ear and Nose Locations*

Parameter \ Location		Ear	Nose
Depth of growth	Prior	3 9	3 2
	Rec	4 4	3 7
	Non rec	3 6	3 6
Loss of palisading	Prior	2 6	2 2
	Rec	3 0	2 3
	Non rec	2 3	2 1
Fibroplasia	Prior	1 8	1 1
	Rec	2 3	1 4
	Non rec	1 5	1 0
Narrow strand	Prior	1 7	1 1
	Rec	2 6	1 6
	Non rec	1 4	0 9
Multifocality	Prior	0 9	0 5
	Rec	1 0	0 6
	Non rec	0 8	0 5
Squamous epithelial differentiation	Prior	0 6	0 7
	Rec	1 0	0 8
	Non rec	0 9	0 7
Cell differentiation	Prior	2 1	2 0
	Rec	2 4	2 1
	Non rec	2 5	2 0

came after X ray treatment and diagnoses were made on limited biopsies

It seems as if X radiation makes the scores higher In 24 tumours scoring was made before and after X ray The mean score before X ray was 11 1 and after 13 6

DISCUSSION

The impetus for this study came from the impression that some basal cell carcinomas of the head and neck region were more aggressive than others and that such tumours were preferably located around the nose and ear We searched for a scoring system to make it possible to judge the aggressiveness of each tumour The literature is rather confusing on this issue and previously no definitive scoring system has been published We therefore suggest a system covering all the impor tant parameters in the histological assessment of the basal cell carcinoma

It is important to create a scoring system easy to use by every pathologist As each interpretation of a specimen is somewhat subjective it would be advantageous to have many criteria On the other hand an extensive system can be too impractical for the pathologist and thus not be accepted in the routine work

Our scoring system is constructed of parameters which express the growth pattern of the tumours such as depth of growth multifocality narrow strands loss of palisading and degree of fibroplasia and by parameters reflecting the cell appearance such as the cell differentiation and degree of squamous epithelial maturity The last parameter was chosen in order to detect the so-called metatypical carcinoma (Sehdam et al 1974)

The material is to some extent selective in that only a part of it consists of new untreated cases a great number are recurrences after earlier treatment with surgery and/or X ray This fact makes it impossible to estimate the frequency of recurrence in this material consequently no comparisons with other case materials can be made in this report

On the basis of our scoring system it is evident that tumours on and around the ear are the histologically most aggressive In accordance with this there are many recurrences seen on the ear

What is the reason for this? The structure of the tissue in this very special localization? A longer case history? The thickness of the skin on the ear is sparse and practically no subcutaneous fat is present These facts can contribute to a higher Clark score The location as such can increase the patient s delay and the time factor could be a reason for the more aggressive behaviour of the tumour The

medical records unfortunately often do not record adequate information about the age of the neoplasm

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ACTION ON VARIOUS EXPERIMENTAL TUMOUR-HOST SYSTEMS OF METHOXY-SUBSTITUTED GLYCEROL ETHERS INCORPORATED INTO THE FEED

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Boeryd B & Hallgren B Action on various experimental tumour host systems of methoxy substituted glycerol ethers incorporated into the feed Acta path microbiol scand Sect. A 88 11-18 1980

formation from Melanoma B16 and two MCA sarcomas was inhibited

Key words Chemotherapy lipids methoxy substituted glycerol ethers tumour growth and metastases

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Lipids are essential constitutents of all cells and major components of all cell membranes Among

Fatty acids have a central role in the nutrition of tumours and for their growth *The metabolic* pathways of lipids in tumour cells are essentially the same as in normal cells These circumstances have raised the question whether chemotherapy interfe ring with fatty acid metabolism could be a possible means of cancer control (*Spector* 1975) However anti tumour activity by lipids has been reported (*Boeryd et al* 1971 *Weitzel et al* 1972 *Ando et al* 1972) It has also been possible to alter the fatty acid composition of an expe

In previous studies the mixture of 2 methoxy *substituted* glycerol ethers isolated from Greenland shark liver oil (*Hallgren and Stallberg* 1967) inhibited metastasis formation from a MCA induced sarcoma (*Boeryd et al* 1971) and in preliminary studies the synthetic compound 1 0-(2 methoxy hexadecyl) glycerol (MGE 16 0) inhibited the growth of some experimental tumours (*Boeryd et al* 1974) Furthermore the methoxy substituted glycerol ethers have been shown to stimulate the plaque forming cell response (PFC) against sheep red blood cells (SRBC) and to increase the ability of *parental* spleen cells to induce graft versus host re *action* (GVHR) (*Boeryd et al* 1975 1978) The stu dies of the effect of methoxy-substituted glycerol ethers (MGE) on experimental tumours have now been extended to include several tumour host *systems* The compounds tested have been mainly synthetic 1 0-(2 methoxy hexadecyl) glycerol MGE 16 0 1-0-(2 methoxy hexadecenyl) glycerol MGE 16 1 and 1 0-(2 methoxy-dodecyl) glycerol MGE 12 0 The mixture of methoxy-substituted glycerol ethers isolated from Greenland shark liver oil was tested in one tumour host system

MATERIAL AND METHODS

Animals and tumours All strains of mice used were inbred In the separate experiments the animals were of similar age and of similar weight, varying between 22–28 g in the different experiments Each group comprised 10–20 mice

The tumours used were in C57BL/6J mice Melanoma B16, Mammary carcinoma Ca 755, Lewis Lung Tumour LLT, Myeloid leukemia C1498 and MCA-induced sarcoma MCG101 (*Boeryd and Suurküla* 1973), in DBA/2J mice Leukemia L1210, Leukemia P388 in ascitic form and Lymphatic leukemia P1534, in A/Sn mice lymphoma LAA, in C3H mice Spontaneous mammary carcinoma MaCaI, in CBA mice MCA-induced sarcoma MCG1 in its solid form, MCG1-SS, in its ascitic form, MCG1-AA, and in its solid ascitic form, MCG1-AS (*Mellgren et al* 1966), MCA-induced sarcoma MCG12 and the MCA-induced epidermoid carcinoma EpCaI (*Suurküla and Boeryd* 1975)

Ca 775 and P388 were obtained from Dr *A Goldin* National Institute of Health, Bethesda, MD, USA, L1210 from Dr *T Connors*, Chester Beatty Research Institute, London, England, and LLT from Dr *K Hellman*, Cancer Chemotherapy Department, Imperial Cancer Research Fund, London, England, LAA from Dr *G Klein*, Institute of Tumour Biology, Stockholm, Sweden, P1534 and C1498 from The Jackson Laboratory, Bar Harbor, Maine, USA

For the transplantation of the tumours standard procedures were adopted The solid tumours and C1498, P1534 and LAA were transplanted with a small piece of tumour tissue subcutaneously into the flank The ascitic tumours P388 and MCG1-AA were transplanted intraperitoneally with 10^5 and 2×10^6 cells, respectively For transplantation of L1210 the spleen of a donor was minced and diluted 1 10 in saline, of this suspension 0 2 ml was given subcutaneously into the flank When the intention primarily was to study influences on spontaneous metastasis formation, the solid tumour was transplanted subcutaneously to the tail (*Hagmar and Boeryd* 1968) By amputation of the tail with the tumour the survival time of the animals could be prolonged Tail tumours including 2 cm of the tail were weighed at amputation

In two tumour-host systems, MCG101 in C57BL/6J mice and MCG12 in CBA-mice, an immunization transplantation resistance test was performed in mice differently fed during immunization (See Tables 7 and 8)

The investigation also included combination treatment with MGE 16 0 and Cyclophosphamide, Methotrexate and Thiotepa of B16, MaCaI P388, MCG1-SS and C1498

All animals were observed during the whole observation period Weight changes and deaths were noted

Feed The methoxy-substituted glycerol ethers were given in the pelleted feed in a concentration of 0 1% 0 25 0 50, 1 0 1 5 or 2 per cent The treatment was usually started the day after tumour transplantation (day + 1) and continued during the whole observation period if not otherwise stated

In a preliminary study, the ability of parental spleen cells to induce GVHR was increased by MGE 16 0 in a concentration of 0 1 per cent in the feed given to the donors 44 days before the test (*Boeryd et al* 1978) Therefore, in some experiments MGE was given about 1 ½ month before tumour transplantation and during the whole observation period Furthermore the substance was given intermittently in a few experiments

The controls were given pelleted feed with the same composition as the test feed but without the glycerol ethers Water was available *ad libitum*

At the end of the observation period the animals were killed by ether The solid tumours were carefully dissected out and weighed In mice transplanted with C1498, P1534 and P388 the survival time was noted The growth of the ascitic tumour MCG1-AA was determined by measuring the total packed cell volume (TPCV) (*Tarnowski and Bross* 1958) At autopsy of animals transplanted with solid tumour, lymph node metastases were noted From some experiments the lungs were prepared for histological investigation according to *Boeryd* (1965), and the incidence of metastases was determined

Differences in tumour weight between groups were tested according to Wilcoxon's two-sample rank test Differences in incidence of metastases between groups were tested by 2×2 contingency table test $P < 0\ 05$ was accepted as significant In the tables, only the experiments which resulted in a change in tumour growth or in spread will be presented

RESULTS

Melanoma B16 The growth of melanoma B16 was inhibited by 0 5% MGE 16 0 in the feed given day + 1 and tended to be inhibited by 2% of the compound given intermittently In a concentration of 0 1%, MGE 16 0 given day –43 tended to stimulate the tumour growth Furthermore, MGE 16 1 and MGE 12 0 in a concentration of 0 5% in the feed inhibited the growth of B16, but at the same time the average body weight of the mice decreased However, feed restriction resulting in a similar body weight change did not influence significantly the tumour growth (Table 1) Spontaneous metastases to the lungs from melanoma B16 transplanted to the tail tended to be inhibited in mice given 0 1% MGE 16 0 for 43 days before tumour transplantation (Table 2)

LLT The growth of this tumour was inhibited by MGE 16 0 given in a concentration of 0 1% 43 days before tumour transplantation while in a concentration of 0 25% it had no effect The incidence of pulmonary metastases was not influenced by the substance in either concentration (Table 3) Treatment of the animals with the substance the day after tumour transplantation did not affect the tumour growth (results not given in tables)

TABLE 1 *Effect on the growth of Melanoma B16 by different Methoxy substituted glycerol ethers*

Groups and treatment	No of animals	Test diet (days)[1]	Observ period (days)	No of mice dead[2]	Average body weight change (g)	Average tumour weight (g)	P
Controls	10		14	1	+0 4	0 93	
0 5% MGE 16 0	10	+1	14		−0 7	0 52	<0 05
Controls	15		18		−0 8	0 36	
2% MGE 16 0	15	+1–4 8–13	18	2	−1 8	0 21	<0 15
Controls	20		21	1	+1 5	1 61	
0 1% MGE 16 0	20	−43	21	2	+0 5	2 13	<0 10
0 25% MGE 16 0	20	−43	21		+2 2	1 56	
Controls	15		17		+0 3	1 10	
0 25% MGE 16 1	15	+1	17		−0 5	0 90	
0 5% MGE 16 1	15	+1	17	1	−3 2	0 61	<0 05
Feed restriction 2 5 g/day	15		17		−2 7	0 91	
Controls	15		17		+1 3	0 8	
0 25% MGE 12 0	15	+1	17		−0 5	0 8	
0 5% MGE 12 0	15	+1	17		−2 9	0 5	<0 05

1) Indication of one day only means the day the treatment started and that it lasted for the whole observation period
2) Mice dead before termination of experiment

MCG101 The growth of this sarcoma tended to be inhibited by 1% MGE 16 0 in the diet given the day after tumour transplantation (Table 3)

LAA The local growth of this tumour was significantly inhibited by 1% MGE 16 0 given the day after tumour transplantation Interval treatment with 2% was also effective (Table 3)

MaCal [illegible]

tion of 2% given intermittently inhibited the growth of the carcinoma (Table 3) The synthetic compound, MGE 16 0 given intermittently during the last 2 weeks of the observation period tended to

TABLE 2 *Effect of MGE 16 0 on Spontaneous Metastasis Formation from Melanoma B16 and MCG1 SS Amputation of Tail Tumours after 30 and 13 Days Respectively*

Tumour	Groups and treatment	No of animal	Test diet (days)	Observ period (days)	Average body weight change	Average tumour weight with 2 cm of the tail	Incidence of metastases to lymph nodes: Gluteal	Incidence of metastases to lymph nodes: Lumbar	to lungs	Total
B16	Controls	25[1]		56	−0 2	0 70	12/23	3/23	15/23	15/23
	0 1% MGE	25[2]	−45	56	+0 8	0 68	12/22	1/22	8/22[3]	13/22
MCG1 SS	Controls	20		24	+1 8	0 20	15/20	10/20	10/20	15/20
	0 5% MGE	20	+1	24	+1 1	0 16	8/20[4]	5/20	5/20	9/20

1 2) 2 and 3 mice respectively died before the end of observation period
3) 8/22 # 15/23 P = 0 10
4) 8/20 # 15/20 P = 0 10

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LLT The growth of this tumour was inhibited by MGE 16 0 given in a concentration of 0 1% 43 days before tumour transplantation, while in a concentration of 0 25% it had no effect The incidence of pulmonary metastases was not influenced by the substance in either concentration (Table 3) Treatment of the animals with the substance the day after tumour transplantation did not affect the tumour growth (results not given in tables)

TABLE 6 *Spontaneous Metastasis Formation from MCG12 after Treatment with MGE 16 0 Animals Allowed to Die of Metastases until Day 156 Amputation of Tail Tumour after 18 Days Treatment Day + 1 − + 18*

Groups and treatment	No of mice	Average tumour weight with 2 cm of the tail	Incidence of spontaneous dead of metastases	of metastases at autopsy after 156 days	of metastases totally
Controls	8	0 71	3/8	1/5	4/8
0 1% MGE	8	1 04[1)]	3/8	1/5	4/8
0 5% MGE	7	0 25[2)]	0/7	0/7	0/7

1) 1 04 # 0 71 $P < 0\ 15$
2) 0 25 # 0 71 $P < 0\ 05$

stimulate the tumour growth This occurred in mice whose average body weight decreased (Table 4) MGE 16 0 given the day after tumour transplantation did not affect the tumour growth (Results not given in the table)

P1534 Some prolongation of the survival time of mice transplanted with this tumour was noted after treatment with 1% MGE 16 0 (Table 5) Lower concentration of the substance had no effect

MCG1-SS The local growth of this tumour transplanted subcutaneously to the tail was not affected by MGE 16 0 However, 0 5% of the substance in the feed tended to inhibit the spontaneous metastases to the regional lymph nodes from tail tumours (Table 2)

MCG12 MGE 16 0 in a concentration of 0 5% inhibited the growth of MCG12 transplanted to the tail None of these mice died of metastases within an

TABLE 7 *Immunization – Resistance Test with MCG 101 Immunization with Tumours Transplanted to the Tail Amputation of Tail Tumours 17 days and challenge 25 Days after Tumour Transplantation Test Diet Days + 1 − + 25*

Challenge cell dose	Incidence of takes after challenge Nonimmunized	Immunized[1)]	1% MGE	Immunized[2)] + 1% MGE
10^7		5/5		5/5
10^6		3/4		5/5
10^5	5/5	3/4	5/5	5/5
10^4	5/5	3/5	5/5	1/5
10^3	5/5	1/5	4/4	0/5
10^2	5/5	2/5	4/4	2/5

1 2) Average weight of tail tumours including 2 cm of the tail 0 45 g and 0 33 g respectively 0 45 # 0 33 $P < 0\ 10$

TABLE 8 *Immunization – Resistance Test with MCG12 Immunization with Tumours Transplanted to the Tail Amputation 21 Days and Challenge 30 days after Tumour Transplantation Test Diet Days + 1 − + 21*

Challenge cell dose	Incidence of takes Nonimmunized	Immunized[1)]	Immunized[2)] + 0 1% MGE	Immunized[3)] + 0 5% MGE
10^5	8/8	7/7	8/8	8/8
10^4	7/8	6/7	7/8	7/7
10^3	7/8	0/4	3/7	3/8
10^2	6/8	0/6	1/7	3/8

1 2 3) Average weight of tail tumours including 2 cm of the tail 0 73 g 0 54 g and 0 33 respectively 0 73 # 0 33 $P < 0\ 05$

TABLE 3 *Effect of MGE 16·0 on the Growth of LLT, MCG 101 and LAA*

Tumour	Groups and treatment	No of mice	Test diet (days)	Observ period (days)	Average body weight change	Average tumour weight	P
LLT	Controls	20[1]		17	+3.7	1.48[2]	
	0.1 % MGE	20	−4–3	17	+3.5	0.97[3]	<0.01
	0.25% MGE	20	−4–3	17	+3.6	1.36[4]	
MCG101	Controls	10		14	+2.3	1.84	
	0.5% MGE	10	+1	14	+2.9	1.43	
	1.0% MGE	10	+1	14	−0.7	1.16	<0.10
LAA	Controls	10		15	+1.5	1.37	
	0.5% MGE	10	+1	15	+1.7	1.06	
	1.0% MGE	10	+1	15	−0.4	0.78	<0.05
	Controls	15		17	+2.1	1.63	
	2% MGE	15	+ 1–5 8–12 15–16	17	−0.3	1.04	<0.01

1) 3 mice died before the end of the observation period
2 3 4) Incidence of pulmonary metastases was 13/17, 18/20, 17/20, respectively

TABLE 4 *Effect of the Mixture of MGE Isolated from Greenland Shark Liver Oil and MGE 16·0 on the Growth of MaCaI*

Groups and treatment	No of mice	Test diet (days)	Observ period (days)	No of mice dead	Average body weight change	Averge tumour weight	P
Groups	20		29		−0.1	0.60	
2.0% MGE_{mix}	20 ·	+1–14 16–21	29		+0.2	0.22	<0.02
Controls	10		31		+2.0	0.34	
1.5% MGE 16.0	10	18–22 26–31	31	1	−1.2	0.69	<0.15
1.5% MGE 16.0	10	15–18 22–26	31	2	+1.2	0.38	
1.5% MGE 16.0	10	18–31	31	1	−2.5	0.71	<0.10

TABLE 5 *Effect of MGE 16·0 on Survival Time of Mice Transplanted with P1534*

Groups and treatment	No of mice	Test diet (days)	Average body weight change on 18th day	Average time (days)
Controls	10		−0.7	18.8
1% MGE	10	+1	−1.3	30.5

Anderson (1977) demonstrated a higher content of ether linked phospholipids in human brain tumour than in normal brain gray matter *Howard et al* (1972) found a correlation between growth and differentiation of transplanted hepatomas and levels of ether linked lipids The most rapidly growing tumours had the highest levels and the less rapidly growing and more highly differentiated ones had progressively lower levels of ether linked lipids The authors also generally found an inverse relationship between the levels of ether lipids and the levels of α glycerol phosphate dehydrogenase

Unsubstituted alkoxylipids have been described as having bacteriostatic properties hematopoetic effects and protective properties against radiation damages (*Mangold* 1972) The effect of methoxy substituted glycerol ethers on biological systems is however totally unknown (*Mangold* 1972) The substitution of the glycerol ethers by a methoxy group might change the activity of the glycerol ethers Unsubstituted glycerol ethers have been described as stimulating certain bacteria (*Brohult* 1960) while methoxy-substituted ones have an antibiotic affect against several bacteria (*Boeryd et al* 1971)

There are probably several mechanisms behind the effect of the methoxysubstituted glycerol ethers on tumour growth and spread, but they cannot be decided on the basis of the present results The substances included in the feed are adjuvants (*Boeryd et al* 1978) and may influence the tumours by immune mediated mechanisms However in some of the experiments discussed above non immunological effects on the tumours seem more reasonable Methoxy substituted glycerol ethers occur normally in man, e g in bone marrow although in trace quantities It might be that the supply of MGE in the feed interferes with host resistance against tumours but not necessarily by immune mechanisms

The host lipid metabolism can be profoundly altered during the growth of tumours both in animals and man (*Costa* 1977) Alteration of the fatty acid composition of experimental tumours cells by changing the content of fat in the diet has also been described (*Rao and Abraham* 1975) The methoxy substituted glycerol ethers may possibly be incorporated into the tumour cell lipids and/or membranes phospholipids (*Friedberg and Halpert* 1978) and thereby change the ability of the tumour cells to grow

In conclusion it has been possible to demonstrate influences although not dramatic ones on tumour growth or spread by lipids normally occurring in man when these substances are incorporated into the feed Depending on the treatment schedules and concentration of the substances the effect varied In a few experimennts the growth of the tumours tended to be stimulated but in most systems affected the growth or spread of the tumours was inhibited.

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observation period of 156 days and at autopsy none of the mice had metastases (Table 6) The growth of MCG12 transplanted subcutaneously into the flank was not influenced by MGE 16 0 in concentrations of 0 1%, 0 25 or 0 5% (Results not given in tables)

In the immunization-resistance test with MCG101 the C57 mice were treated with 1 0% MGE 16 0 until challenge The average weight of the tail tumours tended to be inhibited by MGE but the resistance against the challenge tumour cells in these preimmunized mice was not increased Pretreatment with the same concentration of MGE but without immunization did not affect the incidence of takes from the challenged tumour cells compared to controls In the similar immunization-resistance test with MCG12 in CBA-mice treated with 0 1% or 0 5% MGE 16 0, the growth of the immunizing tail tumour was inhibited by 0 5% of the compound However, the resistance against the challenged tumour cells in these pre-immunized mice given MGE was, if anything, decreased compared to the preimmunized mice not given MGE

The combination treatment with the different chemotherapeutics and MGE 16 0 did not accentuate the effect of any drug included

The tumours not influenced by MGE were Ca755, C1498, L1210, P388, MCG1-AA, MCG1-AS and EpCal

DISCUSSION

For the evaluation of the anti-tumour effects of synthetic methoxy-substituted glycerol ethers, a broad spectrum of tumour-host systems has been used, including carcinomas, sarcomas, lymphomas and leukemias on different inbred strains of mice MGE 16 0 has been tested on all tumour-host systems included, MGE 16 1 on B16 and Ca755, and MGE 12 0 on B16 MaCal and P1534 Of these compounds, MGE 16 0 was most effective and less toxic, and in various concentrations and treatment schedules inhibited the growth of several tumours, although to different degrees Of the systems affected by MGE there seems to be no preference for any type of tumour However, none of the two ascitic tumours tested was influenced

The spontaneous metastases formation tended to be inhibited by MGE 16 0 in three systems In a previous study the mixture of methoxy substituted glycerol ethers inhibited significantly spontaneous metastases to the lymph nodes from MCG1-SS (*Boeryd et al* 1971) In the present study, the synthetic MGE 16 0 tended to inhibit the dissemination of the same tumour to regional lymph nodes

The PFC response to SRBC was stimulated in CBA mice by MGE 16 0 in concentrations of 0 5% in the diet for 4 days before SRBC injection and in C57BL/6J mice in a concentration of 0 1% given for 14 days before SRBC The ability of parental spleen cells to induce GVHR was stimulated by *0 1% MGE 16 0 given for 44 days to spleen cell* donors before the spleen cell transfer (*Boeryd et al* 1978) The PFC response is T cell dependent (*Cantor* 1972) and the GVHR is mediated by T cells (*Gowans et al* 1965) Pre-treatment of mice with 0 1% MGE 16 0 for 43–45 days before tumour transplantation and during the whole observation period inhibited the growth of LLT, tended to stimulate the growth of B16, but tended to inhibit the dissemination of B16 transplanted to the tail This low concentration of MGE 16 0 was effective only when it was given for about 1½ months before tumour transplantation In view of the effect of MGE 16 0 on the GVHR, it seems probable that the effect of the substance on B16 and LLT, at least in part, is immune mediated

On the other hand, in the immunization transplantation resistance test with MCG101, treatment with 1% MGE 16 0 during immunization did not increase the transplantation resistance against MCG101 challenged cells Similar experiments with MCG12 resulted, if anything in a decreased transplantation resistance in mice treated with 0 1% and 0 5% MGE 16 0 during immunization In these experiments, however the treatment started the day after transplantation of the immunizing tumour, *i e* similar to most of the experiments in which the antitumour effects by MGE were studied Thus the results of these transplantation resistance tests do not indicate that the effect of MGE 16 0 on MCG101 transplanted s c in the flank (Table 3) or the protective effect against MCG12 metastases from tail tumour (Table 6) is immune mediated

Fatty acids are required for the growth and division of tumour cells (*Henderson and LePage* 1959, *Spector* 1975) The sources of tumour fatty acids can be i) biosynthesis by the tumour itself (*Medes et al* 1957) ii) the host (*Mays* 1969) and iii) the diet (*Littman et al* 1966)

Non substituted glycerol ethers alkyl and alk-1-enyl ones occur in membrane phospholipids (*Horrocks* 1972) *Snyder and Wood* (1968 1969) found that alkyl- and alk-1-enyl glycerol ethers were significantly higher in several experimental tumours and in human tumours studied than in normal tissues, in which these glycerol ethers occur in minor amounts The amount of ether-linked phospholipids was also higher in the tumours than in most normal tissues Furthermore *Albert and*

Anderson (1977) demonstrated a higher content of ether linked phospholipids in human brain tumour than in normal brain gray matter *Howard et al* (1972) found a correlation between growth and differentiation of transplanted hepatomas and levels of ether linked lipids The most rapidly growing tumours had the highest levels and the less rapidly growing and more highly differentiated ones had progressively lower levels of ether linked lipids The authors also generally found an inverse relationship between the levels of ether lipids and the levels of α glycerol phosphate dehydrogenase

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SENSORINEURAL HEARING LOSS IN A SCANDINAVIAN OLD ENGLISH SHEEPDOG

A Case Report

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Sensorineural hearing loss in a Scandinavian old English sheepdog A case report. Acta path microbiol scand Sect A 88 19-23 1980

Inner ear disease is extremely rare in the old English sheepdog The present case would constitute the second known case in Scandinavia and the first one observed in Norway The morphological investigation revealed complete degeneration of the organ of Corti A single layer of cells was covering the anatomical location of the stria vascularis The dog did not show clinical signs of vestibular dysfunction Hair cells were present in the cristae ampullares and macula utriculi The histopathological features support a genetic basis for the sensorineural hearing loss though other (rare) causes of exogenically induced inner ear degeneration cannot be completely excluded on the morphological findings alone The brain revealed signs of chronic meningitis

Key words Old English sheepdog genetic inner ear degeneration meningitis labyrinth

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Inner ear deafness occurs both in animals and in man and is caused either by genetic defects or environmental factors A detailed analysis of the etiology is often difficult in individual cases In maternal life, disease (viral infections erythroblastosis foetalis and others) can be diagnosed early whereas the identification of genetic penetration of various characteristics requires thorough investigation and an extensive material It is well established that genetic deafness occurs in many animals, including the mouse, guinea pig cat and dog (*Altman 1950*)

Anderson et al (1968) described in detail the inheritance pattern and histopathology/ultrastructure of cochlear deafness in the Dalmatian dog A corresponding study was performed by *Ernstson* (1972) concerning the waltzing guinea pig, in which the vestibular part of the inner ear was also included Otherwise, several reports have been published dealing with various aspects of inner-ear dysfunction in animals and man (*Lurie* 1948, *Bosher and Hallpike* 1965, *Mair and Elverland* 1977)

Inner ear disease is extremely rare in the old English sheepdog *Anniko et al* (1977) analysed the first case of inner ear deafness in Sweden and suspected it to have a hereditary background The present case was found in Norway and would therefore, according to my knowledge, constitute only the second old English sheepdog in Scandinavia with clinical manifestations of complete deafness

MATERIAL AND METHODS

Material

The old English sheepdog subject to this study was 5 months old i e not fully grown at the time of this investigation Prior to sacrifice for morphological analysis of the temporal bones the dog was clinically investigated with regard to the startle reflex signs of

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RESULTS

Cl n cal Obsen ations

At the veterinary examination the animal was found to be healthy except for its hearing defect The hearing was tested with short sound clicks at frequences of 1 2 4 and 8 kHz following stimulation with intensit es of 85 100 125 and 140 dB respectively The startle reflex could not be provoked with any of the stimuli The animal had a normal balance There were no signs of nystagmus at rest or other signs of vestibular dysfunction but nystagmus could be provoked by caloric testing (both sides) The middle ear cavity was morphologi cally normal prior to further handling

Morpholog cal Invest gat on

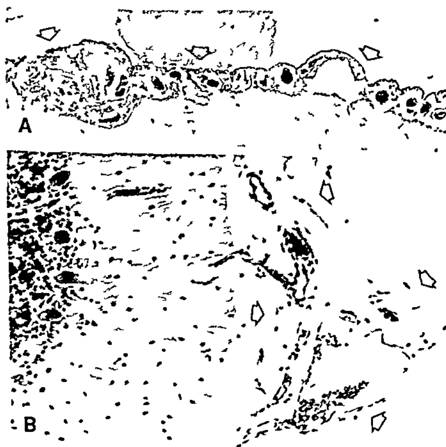
The nner ear Sections were taken from each vest bular organ of the animal and four sect ons from each co l of the two cochleae

Cochlea

In several sections Reissner s membrane had collapsed narrowing the lumen of the cochlear duct It was adherent to the stria vascularis and lay over the remnants of the organ of Corti (Fig 1) In some specimens the degenerated organ of Corti/scar tissue was loosened from the bas lar membrane The tunnel of Corti was collapsed and the tectorial membrane dislodged from the organ of Corti Hair cells could not be identif ed at any level in the cochlea The type of supporting tissue in general could not be identified Gangl on cells were present in great numbers as also were myelinated nerves between the cochlear ganglion and the habenula perforata The stria vascularis consisted of a s ngle cell layer devoid of blood vessels (Fig 2)

Vestibular Organs

Hair cells type I and type II were both identif ed and without obvious s gns to decrease in number



Fg 3 LM *A & B* The men nges (arrows) reveal a m ld accumulat on of h st ocyt c cells ind cative of chron c men ng s 40 × and 120 × respect vely

vestibular dysfunction and of nystagmus at rest and after caloric testing

Methods

Following an intravenous overdose of barbiturate the animal was decapitated and the temporal bones removed The inner ear was reached through an access via the mastoid process and tympanic cavity The basal coil of the cochlea was bulging slightly into the middle ear (containing the auditory ossicles) and it could be easily identified The stapedial footplate was extracted and the round window membrane was removed allowing a gentle perfusion of the perilymphatic space with the fixative a technique often used in inner ear research (*Wersall* 1956 *Anniko* 1978) Thereafter the bone wall of the cochlea was taken away in approximately 1/3 of each coil removing also the stria vascularis to allow a direct flow of the fixative into the endolymphatic partition A hole was drilled in the horizontal semicircular canal to perfuse also the vestibular part of the inner ear For both temporal bones this procedure took less than 5 minutes before fixation a period of time not causing post mortem artefacts even at the ultrastructural level (*Wersall et al* 1965 *Anniko and Bagger-Sjöbäck* 1977)

A solution of 3% glutaraldehyde in 0 133 M sodium phosphate buffer (pH 7 4) was used as the initial fixative (2 hours) followed by postfixation in 2% osmic tetroxid (2 hours) during which the fixative was gently perfused several times through the membranous labyrinth After dehydration in alcohol to 70% the specimens were dissected separating the cochlear and vestibular parts of the inner ear The cochlea and each vestibular organ (3 cristae ampullares utricle and saccule) were separately embedded in epon mixture and sectioned for light microscopy (staining toluidine blue)

The remaining part of the animal underwent ordinary autopsy including fixation of the brain and meninges with 4% formaldehyde (pH 7 4)



Fig 1 Light microscopy (LM) 1st coil of the cochlea Complete degeneration of the organ of Corti Reissner's membrane (indicated by arrows) is depressed obstructing the endolymphatic space and lying close to the stria vescularis (SV) The region between the inner hair cell and the outer hair cells is marked with a filled arrow 140 ×
Inset Higher magnification of the location of the organ of Corti Hair cells cannot be identified 320 ×

Fig 2 LM 2nd coil Stria vascularis of the cochlea A single cell layer covers the anatomical location of the stria vascularis RM Reissner's membrane SP spiral prominence SV stria vascularis 320 ×

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Morphometrical methods were however not used The secretory/re absorptive region around the vestibular organs revealed a normal morphology

The Brain

The brain underwent ordinary autopsy Gross morphologic/ pathologic changes were not found Fifteen pieces comprising brain and adjacent meninges taken from different parts of the cerebrum were analysed at the light microscopic level All specimens revealed an increased thickness of the meninges and also a slight to moderate degree of histocytic cell proliferation indicative of a chronic meningitis (Fig 3A & B)

DISCUSSION

The present study reveals a selective degeneration of the auditory partition of the inner ear leaving the vestibular organs intact This is in agreement with the previous report by *Anniko et al* (1977) describing a similar type of histopathology

According to clinical observations the animal was deaf from birth thus indicating a pre natal inner ear damage but without vestibular dysfunction The stria vascularis was composed of a single layer of undifferentiated cells Such a feature can be observed either in genetic disorders or in inner ear disease later in life (*Anderson et al* 1968 *Schuknecht and Igarashi* 1964) However the correspon ding epithelium around the vestibular organs and also most of the vestibular hair cells had a normal appearance Independent systems for endolymph turnover (production/reabsorption) occur in the cochlea and vestibular organs (*Dohlman* 1964 1965) – which thus can explain a normal/sufficient function of balance

During embryogenesis and cytodifferentiation *hair cells develop completely independently of other* structures (*Anniko et al* 1979) A morphologically developed organ of Corti may precede its degenera tion at a later stage before or after the physiological maturation and function of the organ as a whole In the present case there occurred no signs of partial or complete aplasia of the inner ear thus indicating that the labyrinth has passed its organogenesis before damage took place Either the cochlea had failed to develop hair cells and specialized cells in the stria vascularis during cytodifferentiation or these structures had undergone a regressive degeneration after morphologic differentiation Such a transfor mation can occur both pre natally and after birth Independently of the stage at which injury took place such inner ear pathology is frequently connected with hereditary disorders (*Suga and Hattler* 1970 *Rebillard et al* 1976)

In the present case the whole organ of Corti had disintegrated at the time of investigation so the question of where in the cochlea the process has started cannot be solved A large number of genetic hearing defects begin in the middle range of hearing both in animals and in man (*Johansson et al* 1964 *Änggård* 1965, *Bosher and Hallpike* 1964) in contrast to hair-cell degeneration caused by exogenous agents (*Ylikoski* 1974 *Wersall* 1978 personal communication) with primarily a high frequency loss with damage of the hair cells in the basal part of the cochlea Primary lesions at the apex are extremely few (*Anniko* 1976)

The occurrence of meningitis in the present animal is obscure and might be coincidental However, meningitis with or without involvement of inner ear physiology/morphology has been reported (review *Schuknecht* 1974) It appears unlikely that an infection of the labyrinth would be restricted only to the cochlear partition especially as there are no anatomical or physiological barriers between the auditory and vestibular parts On the contrary, the endolymph is in continuous contact with both parts of the inner ear

In conclusion the histopathologic appearance of the present case strongly supports a genetic basis for the sensorineural hearing loss

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Acta path microbiol scand Sect A 88 25–39 1980

THE ULTRASTRUCTURE OF BONE MARROW PLASMA CELLS OBTAINED FROM PATIENTS WITH MULTIPLE MYELOMA DURING THE CLINICAL COURSE OF THE DISEASE

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Blom J Hansen O Paaske & Mansa B The ultrastructure of bone marrow plasma cells obtained from patients with multiple myeloma during the clinical course of the disease Acta path microbiol scand Sect. A *88* 25–39 1980

The ultrastructure of plasma cells from 65 consecutive bone marrow specimens from 13 patients with multiple myeloma is described Biopsies were taken from 12 of the patients prior to and after initiation of treatment The study was undertaken with the aim of correlating ultrastructural characteristics of the myeloma cells with biochemical and clinical parameters including the survival time after treatment of the patients with cytostatics Intranuclear inclusions were only seen [illegible]
whom had rather long surv[illegible]
are characterized by a low [illegible]
respectively Nuclear bodies [illegible]
treatment was initiated D[illegible]
0 004) A nuclear/cytoplasm[illegible] asynchrony was a characteristic feature of the ultrastructure of the myeloma cells During treatment a significant increase from 34 to 54 per cent (2P = 0 03) was observed in the number of plasma cells with slight asynchrony No correlation could be established between any ultrastructural features and the values obtained with clinical tests considered of major prognostic significance at the time of diagnosis *e g* the concentrations of serum creatinine haemoglobin serum albumin and serum calcium

Key words Plasma cells multiple myeloma ultrastructure nuclear/cytoplasmic asynchrony

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Accepted as submitted 16 vii 79

Several papers (5 23 28) have presented detailed descriptions of the pleomorphic ultrastructure of the bone marrow plasma cells from patients with multiple myeloma The cells have been characterized (16 32) by an asynchronous maturation of nuclear chromatin and cellular cytoplasm However in another study (34) it was concluded that there was no characteristic plasma cell abnormality

The present investigation was initiated with the intention of following a group of patients with multiple myeloma throughout the clinical course of their disease and simultaneously studying the ultrastructure of their bone marrow plasma cells and the concentration of the M components in their sera

The study comprises the examination of 65 bone marrow biopsies and serum samples from 13 patients These patients were examined at intervals throughout the clinical course of their disease and the first biopsy was taken at the time of the diagnosis prior to any treatment with cytostatics

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MATERIAL AND METHODS

The criteria used for the diagnosis multiple myeloma were as follows (18)· 1) Presence of at least 3 per cent atypical plasma cells in a bone marrow smear or a histological proof of a plasmacytoma or either of these conditions combined with a high concentration of a serum M component *i e* IgG ≧ 30 g/l (≧ 373 units/ml) IgA ≧ 20 g/l (≧ 1190 units/ml) or combined with excretion of high concentrations (> 60 mg/l)(9) of light chains in the urine 2) Presence of at least 3 per cent atypical plasma cells in a bone marrow smear or a histological proof of a plasmacytoma or either of these conditions combined with the presence of osteolytic bone lesions on an X ray photograph

Immunochemistry Qualitative immunoelectrophoretic analysis and determination of the concentrations of the serum M components were carried out as described previously (6)

Patients

Clinical and Laboratory Findings

Thirteen patients (5 men and 8 women) with multiple myeloma were studied (Table I) The average age of the men was 58 years (range 41–70) and that of the women 61 years (range 43–76) Five of the patients had an M component of the IgG_1 type lambda* four an IgG_1 kappa one an IgG_2 lambda, one an IgA kappa and two patients had Bence Jones protein of the type kappa in their serum

Seven of the patients (54 per cent) were found to have osteolytic bone lesions when the multiple myeloma was diagnosed The first sign of the disease in two of the patients (OB ES) who had M components of kappa light chains in their sera was a plasmacytoma localized to the lumbar vertebra A plasmacytoma was also found in a third patient (GH) but in that case it was present in the mandible The tumours were surgically removed from patients OB and ES who were subsequently treated by X ray irradiation When the plasmacytoma was discovered in these three patients the disease had disseminated to the bone marrow

Bone marrow material from a patient with no sign of multiple myeloma was also studied in order to obtain some control plasma cells for comparison with plasma cells from [illegible]

wa[illegible]

for [illegible] mmol/l (normal range 8 2–10 9 mmol/l) An elevated serum creatinine concentration was found [illegible] in 31 per cent of the patients with a mean of 178 μmol/l (normal concentration < 120 μmol/l) Initial serum albumin concentrations were decreased in 77 per cent of the patients with a mean concentration of 0 39 mmol/l (normal range 0 56–0 83 mmol/l) The values for the total serum calcium concentration after correction for the lowered concentrations of serum albumin were found to be increased in 31 per cent of the patients but only one (JN) showed a definite hypercalcaemia with a value of 4 13 mmol/l (normal range 2 27–2 55 mmol/l)

The Period of Observation

The patients were examined at intervals during the course of their disease (Table I) Two patients are still alive During the period of observation a total number of 65 bone marrow aspirations (range 2–15 per patient) were examined The first bone marrow sample from a patient was taken prior to the initiation of treatment with cytostatics (melphalan or cyclophosphamide) Although one patient (EH) had been treated with melphalan before the first biopsy was taken she was included in the study because she had Russell bodies in her plasma cells (6)

Methods for Electron Microscopy

The methods used for fixation and further preparation for electron microscopy of the bone marrow aspirations are described previously (6) Sections were made of three or more marrow particles from each of the 65 biopsies The number of plasma cells on the individual micrographs of our survey fields at a magnification of 15 000 × varied from specimen to specimen but a mean number of 25 different plasma cells from each biopsy was chosen for evaluation A total number of 1600 plasma cells was selected from this material for further evaluation The cells were selected so that they contained an abundance of the rough endoplasmic reticulum and were sectioned through their central region in such a way that the nucleus was included in the section In most cases the Golgi complex was also present in cells sectioned in this way The selection of cells for the evaluation was made by a technician considered to be less biased than any of the authors Evaluation of the micrographs was carried out by one of the authors (JB) who was unaware of the results of the laboratory tests at this stage of the study Approximately 5000 electron micrographs were examined

Plasma Cell Evaluation

Each plasma cell included in a section was evaluated at a magnification of 15 000 × and for more detailed study at 90 000 × The following parameters were taken into account

[illegible]

cisternae of the rough endoplasmic reticulum was registered as a PS

Rough endoplasmic reticulum (ER) It was noted whether the reticulum was of lamellar or globular type Intracisternal bodies (*Russell bodies*) were registered

Mitochondria Number was counted and length and diameter measured when possible

Cytoplasmic inclusions Number was counted and diameter measured

Golgi complexes Number of centrioles in Golgi complexes was registered

Contact zones Number of these between plasma cells

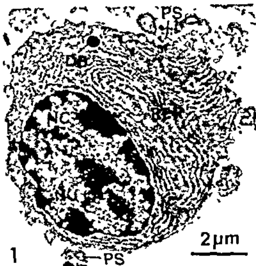
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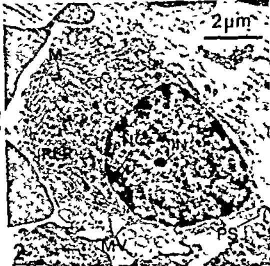
* The IgG subclasses were kindly determined by Dr S Skvaril Bern Switzerland

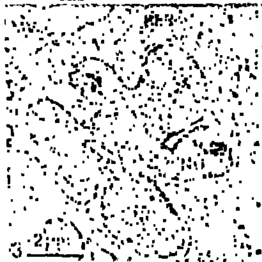
TABLE 1 *Results of Laboratory Tests Performed at the Time when the First Bone Marrow Biopsy was Taken*

Patient	Age/sex	Serum monoclonal protein	Serum conc of M component (units/ml)[b]	HB (mmol/l)[b]	Se creatinine (μmol/l)[b]	Se calcium (mmol/l)[b]	Se albumin (mmol/l)[b]	Survival *time* (months)	% plasma cells *of nucleated cells* in bone marrow
GH[a]	47/M	IgG_1 κ	341	8 0	199	2 71	0 46	2 5	13
MN[a]	68/F	IgG_1 λ	1129	5 6	113	2 32	0 37	3 5	31
LB	76/F	IgG_1 λ	729	5 9	200	2 38	0 49	7	28
VS[a]	67/M	IgG_1 λ	1258	6 5	143	2 59	0 27	13	61
KC	75/F	IgG_1 κ	1255	5 1	120	2 35	0 47	20	20
CB[a]	67/M	IgG_1 κ	971	6 8	87	2 67	0 28	24	66
EJ	43/F	IgG_1 λ	690	6 8	121	2 39	0 42	29	46
JN[a]	63/F	IgG_2 λ	416	3 3	87	4 13	0 35	29	87
EH	60/F	IgA κ	2610	4 9	170	2 55	0 47	29	24
GC	47/F	IgG_1 λ	662	7 8	87	2 51	0 61	53	22
OB	41/M	Kappa	c	8 6	104	2 50	0 62	>62	10
ES[a]	70/M	Kappa	c	9 0	116	1 36	0 59	63	5
AS[a]	54/F	IgG_1 κ	775	7 8	87	1 63	0 32	>78	30

[a] Osteolytic lesions
[b] Normal range see Material and Methods
[c] 1–2 g/l

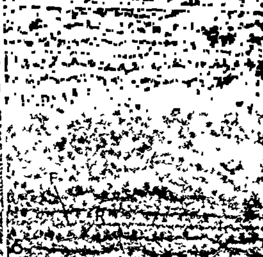

PS
DB
NC
2μm
1
PS


2μm
M
RER
PS
2


3




2μm
5


6

and dendritic bone marrow macrophages was counted (see ref 8 for details)

Shape of the nucleus Round ovoid or irregular

Nucleoli Number was counted and diameter measured

Nuclear inclusions and nuclear bodies Numbers were counted (for a detailed definition see ref 15 and later in the text) and structures examined in detail

Nuclear/cytoplasmic asynchrony *Bernier & Graham* (3) have described nuclear/cytoplasmic asynchrony of plasma cells at the ultrastructural level and their classification (slight moderate or marked asynchrony) has been used throughout this study

For the individual patients the incidence of the different parameters was registered in all the plasma cells selected and for each biopsy the percentage of each parameter was estimeted and compared with the result obtained for the other biopsies

Statistics

The Wilcoxon test for pair differences was used (14) for comparison of the numbers of nuclear bodies found in individual plasma cells from untreated and treat patients and for the evaluation of slight asynchrony

Text to Figures

Except for Fig 1 all figures show electron micrographs of sections obtained from bone marrow specimens from patients with multiple myeloma The bar on each micrograph represents 100 nm unless otherwise stated

RESULTS

The ultrastructure of 1600 plasma cells from tl bone marrow of the 13 myeloma patients wa studied according to the principles mentioned ar considerable variation from patient to patient wa found In contrast, the morphology of the cel appeared to change very little for each individu patient during the clinical course of the disease

Most of the cells had a round or ovoid shape wit slender, thin microvilli like extensions (MV) on th surface (Figs 2, 3, 17) and frequently also a sma number of blunt cytoplasmic projections or pseudo pods (PS) (Fig 2) In one patient (OB) > 30% c the cells showed PS in every biopsy and on thes cells the PS were more frequent than the MV Fo this particular patient – who is still alive – the M component of the serum was found to contain ligh chains only

Rough Endoplasmic Reticulum

The cisternae of the rough endoplasmic reticulun (ER) in the plasma cells from individual patient usually presented one of two morphological types

Fig 1 A survey of a bone marrow plasma cell from a patient without any sign of multiple myeloma The nuclear chromatin (NC) shows a characteristic peripheral clumping and a small nucleolus (NU) A parallel arrangement of the cisternae of flattened rough endoplasmic reticulum (RER) is seen in the cytoplasm There is no evidence of nuclear cytoplasmic asynchrony On the cell surface a few pseudopod like (PS) extensions of the cytoplasm are seen DB denotes a dense body 7 500 ×

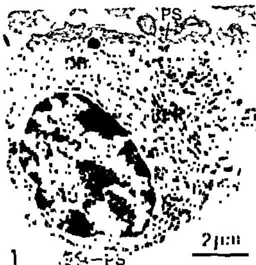
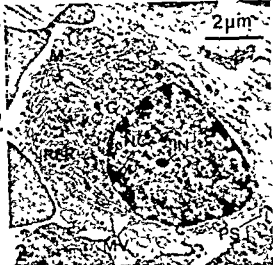
Fig 2 A survey of a plasma cell from patient AS The nuclear chromatin (NC) shows small areas of condensation distributed through the nucleus and only slight aggregation at the periphery An intranuclear inclusion (IN) is also present This is given in higher magnification in Fig 16 The cytoplasm contains parallel cisternae of the rough endoplasmic reticulum (RER) and several mitochondria (M) are seen around the well developed Golgi complex (G) This cell demonstrates slight nuclear cytoplasmic asynchrony Microvilli like (MV) as well as pseudopod like (PS) extensions are present on the cell surface 7 500 ×

Fig 3 A survey of a part of a plasma cell from patient VS The nucleus is very irregular and the nuclear chromatin (NC) is well dispersed with little or no aggregation A small eccentrically located nucleolus (NU) is present The cytoplasm contains parallel cisternae of the rough endoplasmic reticulum (RER) and several mitochondria (M) are seen This cell demonstrates a moderate nuclear cytoplasmic asynchrony MV denotes microvilli like extensions 7 500 ×

Fig 4 A survey of a part of a plasma cell from patient EJ The nucleus appears immature with well dispersed non aggregated chromatin A moderately large nucleolus (NU) is present The mature well differentiated cytoplasm contains an abundance of dilated cisternae of the rough endoplasmic reticulum (RER) Russell bodies (Rb) are found in a few of the RER cisternae A well developed Golgi complex (G) is seen together with several mitochondria (M) This cell demonstrates marked nuclear cytoplasmic asynchrony 7 500 ×

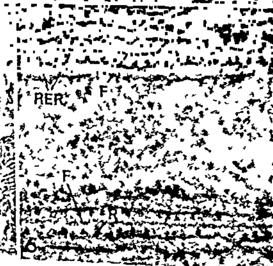
Fig 5 A survey of a plasma cell from patient OB A longitudinal section through a concentric arrangement of the rough endoplasmic reticulum (RER_1) is seen This structure is situated close to the Golgi complex (G) A similar structure (RER_2) is shown in cross section close to the nucleus A nucleolus (NU) of moderate size is present in the slightly irregular nucleus The cell presents slight nuclear-cytoplasmic asynchrony 7 500 ×

Fig 6 High power view of part of the RER_1 from the plasma cell shown in Fig 5 Note filaments (F) between the parallel cisternae of the rough endoplasmic reticulum (RER) and their contact with the ribosomes (R) of the reticulum 90 000 ×


PS
2 µm
1
PS

2µm
M
G
RER
NU
PS
2

3
2µm

2µm
4

RER
NU
2µm

RER
F
F
6

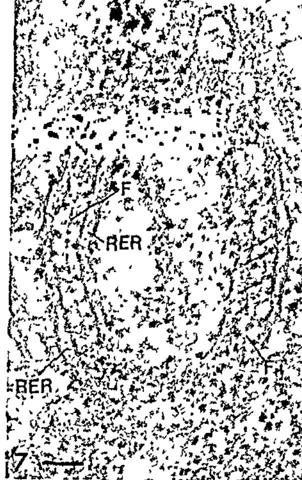








Fig 7 Part of a plasma cell from patient OB A concentric arrangement of two cisternae of the rough endoplasmic reticulum (RER) is seen Note the filament (F) located in the cytoplasm between the cisternae and its contact with the ribosomes (R) of the RER at certain points (arrows) 90 000 ×

Fig 8 Part of a plasma cell from patient ES The cisternae of the annulate lamellae (AL) are continuous with the cisternae of the rough endoplasmic reticulum (RER) AN denotes the annuli formed where fusion of the paired membranes of the cisternae of the annulate lamellae has occurred 90 000 ×

Fig 9 Part of a plasma cell from patient FH showing an abnormal mitochondrion with a concentric arrangement of the cristae 90 000 ×

Fig 10 Part of a plasma cell from patient AS showing a dense body (DB) limited by a unit membrane (UM) The DB is an inhomogeneous structure composed of an amorphous and a crystalline region (CS) together with a lipid like inclusion (L) RER denotes rough endoplasmic reticulum 90 000 ×

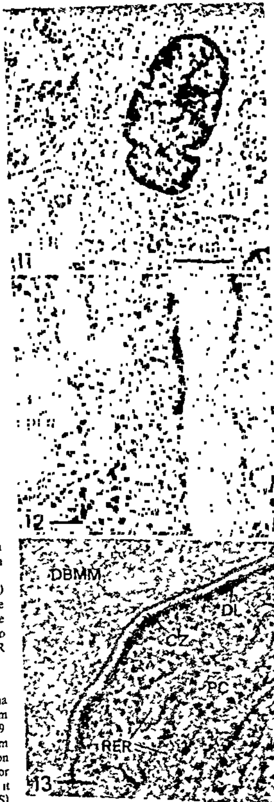
... of a plasma cell from patient MN The cell

...mina Dilated cisternae of the rough endoplasmic reticulum (RER) are also seen 7 500 ×

Fig 12 High power view of part of the plasma cell

...he plasma cell and the endothelial cell Note the presence of vesicles (V) in the endothelial cell and the rough endoplasmic reticulum (RER) with dilated cisternae in the plasma cell 90 000 ×

Fig 13 Part of a plasma cell from patient JN A contact zone (CZ) between the plasma cell (PC) and a cytoplasmic extension of a dendritic bone marrow macrophage (DBMM) is seen Note the dense layer (DL) attached to the inner leaflet of the cell membrane of the plasma cell in the contact region RER denotes rough endoplasmic reticulum 90 000 ×



regular array of parallel, flattened cisternae (Figs 2 3 5 17) or a globular type with dilated cisternae (Figs 4 11) The former was the most common whereas the latter was found in most of the cells from four patients (MN, EJ, EH, OB) and in three of these (EJ, EH, OB) the cells contained Russel bodies (Fig 4), i e electron-dense structures situated within the cisternae of the rough ER (6)

In addition to the rough ER some plasma cell contained different unusual structures situated i the cytoplasm close to the ER Some cells from tw of the patients (MN, ES) showed structure resembling annulate lamellae (21) (Fig 8) they were often found in direct continuation of the rough ER In four patients (KC CB OB AS) there was an unusual circular arrangement of the rough ER (Fig 5) which was seen both in cross sections (Figs 5 7) and in longitudinal sections (Figs 5, 6) In the cytoplasmic matrix between the individual cisternae of the rough ER one to three filaments were seen to be in contact with the ribosomes of the rough ER (Figs 6 7)

Mitochondria

The mean number of mitochondria in the plasma cell sections of individual biopsies varied from patient to patient with a range from 7 to 39 whereas the variation from biopsy to biopsy from each patient was very small Any correlation between the number of mitochondria and clinical or biochemical findings was not apparent However it should be noted that two of the patients (OB AS) have lived for a long period after confirmation of the

diagnosis and both show a low mean number of mitochondria, *i e* 7 and 14, respectively The diameter of the mitochondria was rather constant, between 0 3–0 4 µm, whereas the length varied from 0 5–2 0 µm Only five of the patients (GH, VS, CB, EH, ES) showed morphological abnormalities in a few mitochondria in their plasma cells, where longitudinal, parallel or concentric arrangements of the cristae were present (Fig 9) These changes were found throughout the clinical course of their disease

Cytoplasmic Inclusions

Electron-dense bodies surrounded by a smooth membrane were observed in the cytoplasm of numerous plasma cells from nearly all patients (Fig 17) The diameter of these inclusions varied from 0 5–1 0 µm Often the dense core of the bodies presented a regular or crystalloid substructure and some lipid like material could also be seen (Fig 10)

Golgi complexes

A well-developed Golgi complex was found in most of the plasma cells (Figs 2, 4, 5) The usual components – stacks of flattened sacs, vesicles and vacuoles – were seen together with granules of variable size, shape and density One or more centrioles were observed in about one third of the Golgi complexes studied

Contact Zones

As previously published (7, 8), a number of contact zones between plasma cells and dendritic bone marrow macrophages were seen in biopsies of all of the patients with multiple myeloma (Fig 13) Only one patient (VS), in whom the nuclei of the plasma cells were markedly irregular (Fig 3), had a low number of such contact zones In some patients (MN, LB, CB, OB) contact zones were seen in association with a strip of basement membrane or lamina densa like material instead of the contact with a macrophage extension (Fig 11) Plasma cells presenting this type of zone were often situated in the perivascular compartment of the bone marrow (Figs 11, 12)

Nuclei

Seventy-five per cent of the plasma cells studied had round or ovoid nuclei most of which were eccentrically located in the cytoplasm (Figs 2, 4, 17) In three patients (VS FH OB), more than 50 per cent of the cells in the first biopsy showed a markedly irregular shape of the nucleus (Figs 3, 5) These cells were often characterized by having many cytoplasmic filaments and microtubules localized to the cytoplasmic invaginations of the nuclei (Fig 14) Binucleate plasma cells were seen in 2 per cent of the cells

Nucleoli with a diameter from 0 7–1 9 µm were found in about 40 per cent of the plasma cells (Figs 3, 4, 5) Neither morphology nor size of the nucleoli could be correlated with any of the clinical or biochemical parameters studied

Nuclear inclusions were found in the nuclei of cells from four of the patients (EJ, EH, OB AS) Plasma cells from three of these contained Russell bodies in the nuclei as described earlier (6) Such Russell bodies were characterized by their homogeneous structure and their being surrounded by a single unit membrane (Figs 10, 11 in ref 6) The cells of the fourth patient (AS) had two kinds of intranuclear inclusions *i e* a crystalline and a fibrillar type (Figs 2, 15, 16), both without a limiting membrane These types were found in all of the 15 biopsies obtained from this patient The crystalline type (Figs 15, 21) appeared as a lattice of tubular structures each with an outer diameter of 11-12 nm The fibrillar type consisted of a bundle of almost parallel fibrils, each with a diameter of 9–11 nm (Fig 16)

In this report the term »nuclear bodies« is used to describe a group of intranuclear globules with *different types of substructure (15)* The diameter of these globular structures varied from 0 3–0 7 µm In most nuclei they were easily recognized because of a surrounding clear halo (Fig 17) Three morphologically distinguishable types were found 1) a type composed of fibrils arranged in a concentric formation (Fig 18) 2) a type of a fibrillogranular body consisting of a few small granules surrounded by randomly arranged fibrils (Fig 19) and 3) a type with a dense core surrounded by fibrils (Figs 17, 20) All fibrils found in the nuclear bodies were 6–8 nm in diameter The two first types were the most common Some plasma cells contained two nuclear bodies which generally belonged to the same morphological type The nuclear bodies did not seem to be structurally related to the nucleoli although a nuclear body and a nucleolus were often found within the same nucleus Occasionally, nuclear bodies were seen to surround the crystalline (*Fig 21*) *or fibrillar nuclear inclusions* previously described to be present in the cells of the patient (AS)

Table 2 shows a comparison of the percentage of nuclear bodies in the plasma cells obtained from the patients before and during their treatment During treatment this percentage increased from 7 to 16 which is significant (2P = 0 004) The three different morphological types of nuclear bodies were seen in the cells from the patients both before and during their treatment and no difference in size



Fig 14 Part of a plasma cell from patient VS who had very lobulated nuclei (cf Fig 3) The perinuclear space (PNS) is shown at a region of two lobules Note the transversally cut filaments (F) and microtubules (MT) present in the cytoplasm within the lobules NC denotes nuclear chromatin 90 000 ×

Fig 15 Part of a plasma cell nucleus from patient AS The crystalline type of an intranuclear inclusion is shown NC denotes nuclear chromatin 90 000 ×

Fig 16 High power view of part of the plasma cell nucleus shown in survey in Fig 2 The fibrillar type of an intranuclear inclusion is shown in detail 90 000 ×

Fig 17 A survey of a plasma cell from patient AS This cell shows slight nuclear-cytoplasmic asynchrony Several microvilli like extensions (MV) of the cytoplasm are observed and a dense body (DB) and several mitochondria (M) are present in the cytoplasm Note the nuclear body (NB) surrounded by a clear halo within the nuclear chromatin (NC) 7 500 ×

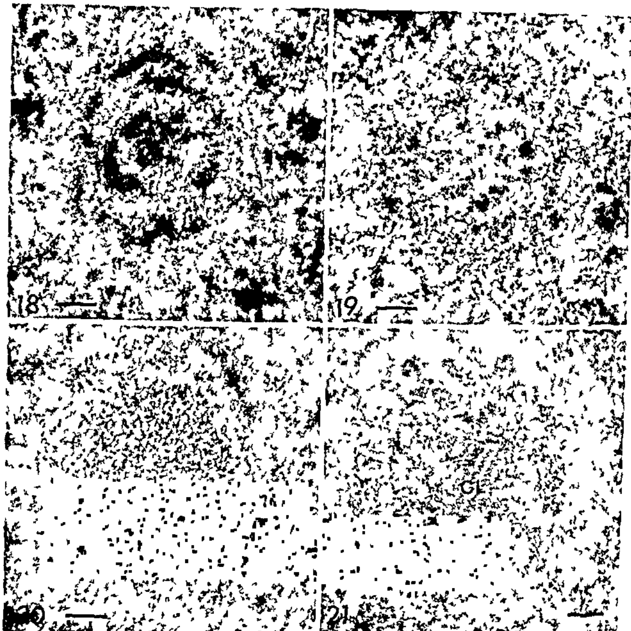


Fig 18 Part of a plasma cell nucleus from patient OB A nuclear body composed of fibrils arranged concentrically is shown 90 000 ×

Fig 19 Part of a plasma cell nucleus from patient JN A nuclear body composed of small granules surrounded by fibrils is shown 90 000 ×

Fig 20 Part of the nucleus of the plasma cell seen in Fig 17 A nuclear body with a central electron dense mass surrounded by fibrils is shown 90 000 ×

Fig 21 Part of a plasma cell nucleus from patient AS A nuclear body surrounding a crystalline intranuclear inclusion (CL) is shown 90 000 ×

was observed in relation to survival time Further more the presence of nu ear bodies had no relationship to the degree of nuclear/cytoplasmic asynchrony

Nuclear Cytoplasmic Asynchrony

Asses ment of the asynchrony by the criteria described n Material and Methods is illustrated by the following figures A plasma cell *without* asynchrony is shown in Fig 1 The cell contains a mature looking nucleus with chromatin clumped in a cartwheel like arrangement Parallel cisternae of the rough ER are seen in the mature appearing well organized cytoplasm Occasionally nucleoli of moderate size are seen in the cells without asynchrony Plasma cells with *slight* asynchrony

TABLE 2 *The Frequency of Nuclear Bod es in Plasma Cells from Patients before and during Treatment*

Plasma cells from 12 patients	Total number of plasma cells	Number of sections of plasma cell nuclei with nuclear bodies	% nuclear bodies of plasma cells (range)
Before treatment[a]	298	20	6 7 (0–13 3)
During treatment	1217	192	15 8 (8 2–23 3)

[a]One patient (EH) is omitted due to treatment at the time of the first biopsy
The Wilcoxon test for pair differences was used untreated versus treated (14) (2P = 0 004)

(Figs 2 5 17) have a mature appearing well organized cytoplasm with parallel stacks of rough ER and a nucleus with scattered small aggregates of chromatin Nucleoli of moderate size are seen in many of these cells Cells with *moderate* asynchrony (Fig 3) show a nucleus with small amounts of chromatin aggregates and a cytoplasm containing stacks of flattened rough ER In some cells with *moderate* asynchrony a nucleus with similar distri bution of chromatin is present together with dilated rough ER in the cytoplasm Cells with *marked* asynchrony (Fig 4) show a nucleus with evenly distributed rather electron lucent chromatin and a cytoplasm containing dilated cisternae of the rough ER [illegible] be

[illegible] re evaluated with respect to nuclear/cytoplasmic asyn chrony it was found that cells without asynchrony were seen only occasionally in the patients Marked asynchrony however was found in cells of the first biopsy from seven of the patients (GH MN CB EJ JN EH AS) After treatment was initiated marked asynchrony could be found only in a small [illegible]

[illegible] that the cisternae of the rough ER were dilated and accordingly the plasma cells were most frequently classified as cells with marked asynchrony Slight asynchrony (Figs 2 5 17) was seen in 34 per cent of the cells from the untreated patients A total number of 298 cells were studied An equal number of cells (292) obtained from the last biopsy of the same patients obtained during treatment showed *that this* percentage had increased to 54 *i e* a significant increase (2P = 0 03)

Clin cal Staging and Course

No correlation could be found between the variation within the intracellular structures found in the plasma cells of the patients and the clinical values of the major tests with prognostic signifi cance obtained at the time of diagnosis *i e* the concentrations of serum creatinine haemoglobin serum albumin and serum calcium All the patients were treated with alkylating agents (melphalan or cyclophosphamide) but the schedules used for individual patients differed to some extent Nine patients had a lower M component concentration in serum samples taken during the treatment than at the time of diagnosis This decrease was most evident for patients with a *short survival time* (MN LB VS KC) as described previously (17) For five patients the irregular intervals (2–26 months) at which blood samples were obtained did not permit a time related evaluation of changes in the M component concentration Serum samples from three patients (KC AS GC) showed a marked increase in the serum concentration of the M component during the course of the disease Plasma cells from patients who developed either a decrease or an increase of the M component showed no evident morphological changes as compared to the plasma cells obtained at the time of diagnosis except for the described increase in the number of nuclear bodies

Six of the patients (MN VS KC, GH JN AS) were treated intermittently with cytostatics intrave nously in doses which were calculated in relation to the body weight of the patients *i e* melphalan 30–50 mg cyclophosphamide 1000–2000 mg The plasma cells obtained from these patients 1 to 30 days after treatment were evaluated and their ultrastructure was compared with that of cells from the [illegible]

[illegible] tage of plasma cells showing slight nuclear/cytoplasmic asynchrony An increase in the percentage of cells with nuclear bodies was also noted

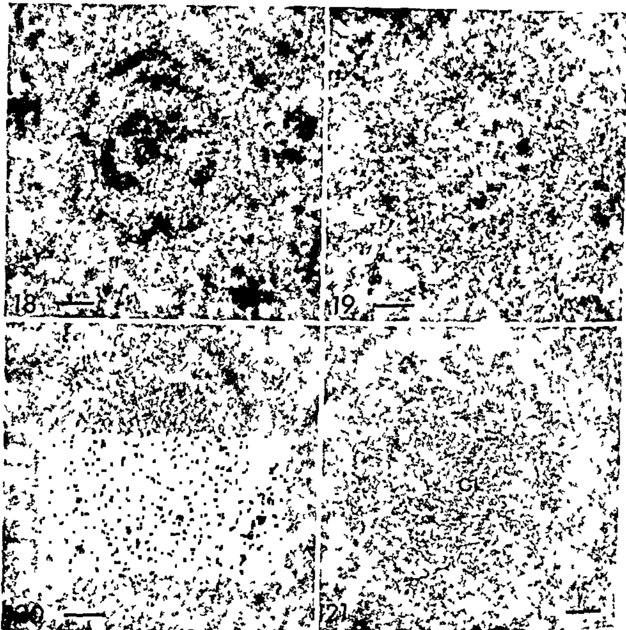


Fig 18 Part of a plasma cell nucleus from patient OB A nuclear body composed of fibrils arranged concentrically is shown 90 000 ×

Fig 19 Part of a plasma cell nucleus from patient JN A nuclear body composed of small granules surrounded by fibrils is shown 90 000 ×

Fig 20 Part of the nucleus of the plasma cell seen in Fig 17 A nuclear body with a central electron dense mass surrounded by fibrils is shown 90 000 ×

Fig 21 Part of a plasma cell nucleus from patient AS A nuclear body surrounding a crystalline intranuclear inclusion (CL) is shown 90 000 ×

was observed in relation to survival time Further more the presence of nuclear bodies had no relationship to the degree of nuclear/cytoplasmic asynchrony

Nuclear/Cytoplasmic Asynchrony

Assessment of the asynchrony by the criteria described in Material and Methods is illustrated by the following figures A plasma cell *without* asynchrony is shown in Fig 1 The cell contains a mature looking nucleus with chromatin clumped in a cartwheel like arrangement Parallel cisternae of the rough ER are seen in the mature appearing well-organized cytoplasm Occasionally nucleoli of moderate size are seen in the cells without asynchrony Plasma cells with *slight* asynchrony

TABLE 2 *The Frequency of Nuclear Bodies in Plasma Cells from Patients before and during Treatment*

Plasma cells from 12 patients	Total number of plasma cells	Number of sections of plasma cell nuclei with nuclear bodies	% nuclear bodies of plasma cells (range)
Before treatment[a]	298	20	6 7 (0–13 3)
During treatment	1217	192	15 8 (8 2–23 3)

[a] One patient (EH) is omitted due to treatment at the time of the first biopsy
The Wilcoxon test for pair differences was used untreated versus treated (14) (2P = 0 004)

(Figs 2, 5, 17) have a mature appearing, well-organized cytoplasm with parallel stacks of rough ER and a nucleus with scattered small aggregates of chromatin Nucleoli of moderate size are seen in many of these cells Cells with *moderate* asynchrony (Fig 3) show a nucleus with small amounts of chromatin aggregates and a cytoplasm containing stacks of flattened rough ER In some cells with *moderate* asynchrony a nucleus with similar distribution of chromatin is present together with dilated rough ER in the cytoplasm Cells with *marked* asynchrony (Fig 4) show a nucleus with evenly distributed, rather electron lucent chromatin and a cytoplasm containing dilated cisternae of the rough ER Prominent nucleoli are seen frequently in the cells showing moderate and marked asynchrony

When all the plasma cells available were evaluated with respect to nuclear/cytoplasmic asynchrony it was found that cells without asynchrony were seen only occasionally in the patients Marked asynchrony, however, was found in cells of the first biopsy from seven of the patients (GH, MN CB EJ JN EH, AS) After treatment was initiated marked asynchrony could be found only in a small percentage of plasma cells in four of these seven patients (MN, CB, EH EJ) Cells from two of them (EH EJ) contained Russell bodies, which means that the cisternae of the [illegible]

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No correlation could be found between the variation within the intracellular structures found in the plasma cells of the patients and the clinical values of the major tests with prognostic significance obtained at the time of diagnosis, *i e* the concentrations of serum creatinine, haemoglobin, serum albumin and serum calcium All the patients were treated with alkylating agents (melphalan or cyclophosphamide), but the schedules used for individual patients differed to some extent Nine patients had a lower M component concentration in serum samples taken during the treatment than at the time of diagnosis This decrease was most evident for patients with a short survival time (MN, LB, VS KC) as described previously (17) For five patients the irregular intervals (2–26 months) at which blood samples were obtained did not permit a time related evaluation of changes in the M component concentration Serum samples from three patients (KC, AS, GC) showed a marked increase in the serum concentration of the M component during the course of the disease Plasma cells from patients who developed either a decrease or an increase of the M component showed no evident morphological changes as compared to the plasma cells obtained at the time of diagnosis, except for the described increase in the number of nuclear bodies

Six of the patients (MN, VS, KC, GH, JN, AS) were treated intermittently with cytostatics intravenously in doses which were calculated in relation to the body weight of the patients, *i e* melphalan 30–50 mg, cyclophosphamide 1000–2000 mg The plasma cells obtained from these patients 1 to 30 days after treatment were evaluated and their ultrastructure was compared with that of cells from the first biopsy The only common morphological difference observed, irrespective of the time interval after initiation of treatment, was a definite increase in the percentage of plasma cells showing slight nuclear/cytoplasmic asynchrony An increase in the percentage of cells with nuclear bodies was also noted

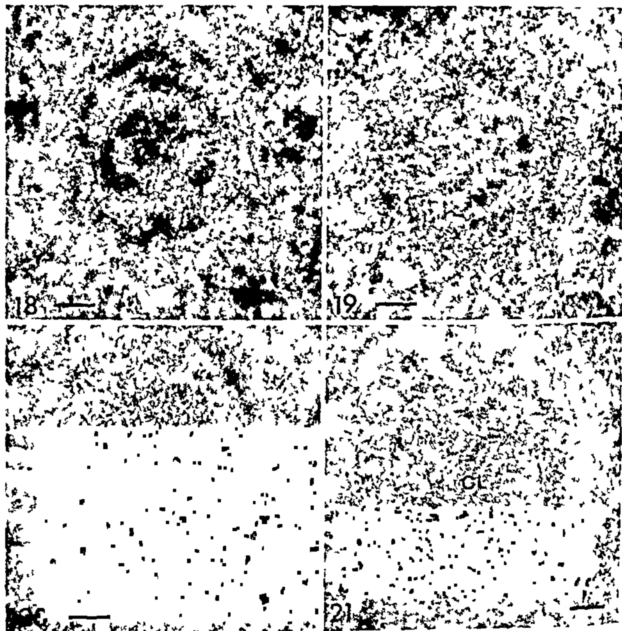


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was observed in relation to survival time Furthermore the presence of nuclear bodies had no relationship to the degree of nuclear/cytoplasmic asynchrony

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shown that their occurrence is not restricted to myeloma cells as contact zones have been found in plasma cells from patients without a serum M component (Blom, unpublished work) Our earlier hypothesis that the contact zones were most frequent in the bone marrow of patients at the terminal stage of their disease (8) could not be corroborated in this study Another type of contact zones was observed between bone marrow plasma cells and a strip of basement membrane or a lamina densa like material Plasma cells with such contact zones were often seen close to a small vessel A structurally similar intimate contact with small vessels has been described in perivascular plasma cells of brain tissue from patients with multiple sclerosis (26)

Only a few myeloma cells in this study showed nuclei with markedly irregular contours It has been suggested that such a nuclear lobulation is produced by a contraction of the microfibrils and microtubules which are accumulated in close association with the nucleus at the region of a nuclear lobulation (2 4)

Three different morphological types of intranuclear inclusions were observed in the biopsies from four of our patients and it should be noted that these patients all had a rather long survival time The intranuclear Russell body was seen in three patients and this observation has been described in detail previously (6) A fourth patient (AS) had two kinds of intranuclear inclusions a crystalline and a fibrillar The fibrillar type has been described in myeloma cells by *Smetana et al* (30) who called them rodlets and by *Oikawa* (25) The crystalline type has to the best of our knowledge not been described before in any plasma cell The nature and significance of these nuclear inclusions are unknown

Nuclear bodies were first described by *De Thé et al* (10) in tumour cells from a rabbit Since then these structures have been observed in several different types of both normal and tumour cells (15) Some papers have described these globular structures in plasma cells (19 29) but only a few observations on their presence in myeloma cells have been made (1 31) The morphology and size of the nuclear bodies found in our study were identical to those already found (e g ref 1 15) Experiments with antigenically stimulated mice showed that the percentage of sections of lymphnode plasma cells containing one or more nuclear bodies increased parallel to the concentration of circulating antibodies (19 29) This result led to a presumed relationship between the number of nuclear bodies in plasma cells and the concentration of serum antibodies *Simar* (29) demonstrated by enzyme digestion of plasma cell sections that the fibrillar component of nuclear bodies contains RNA, whereas the dense granular component contains DNA

Recent studies by means of high resolution autoradiography have shown that nuclear bodies in spleen cells of immunized mice incorporated significantly more radioactive material than the surrounding nucleoplasm (13) *Drewinko et al* (11) and *Salmon* (27) have performed growth kinetic studies on myeloma cells They measured the labelling index (LI) of bone marrow myeloma cells as an indication of the fraction of cells which synthesized DNA and thus could be considered to be a proliferating subpopulation of the myeloma cells It was found that the LI of the myeloma cells increased from approximately 3 per cent before treatment to within the range of 15–40 per cent after administration of alkylating agents to the patients In a later paper, however, *Drewinko & Alexanian* (12) concluded that the LI may not accurately reflect the growth fraction of the tumour mass in patients with multiple myeloma On the other hand, that study showed that the LI for patients observed during the first 3 months of treatment was significantly higher than for untreated patients but after longer periods of treatment the LI declined In our material we noted an increase in the number of nuclear bodies in the plasma cells during treatment of the patients (Table 2) Therefore it seems likely to us that it is the plasma cells with nuclear bodies which are in a proliferative phase If so it should be possible to demonstrate a correlation between the number of nuclear bodies and the LI of the myeloma cells

Snapper & Kahn (32) have considered that the immaturity of the nucleus in relation to the cytoplasm seen in myeloma cells is of diagnostic value *Graham & Bernier* (16) classified nuclear/cytoplasmic asynchrony from ultrastructural studies on plasma cells and concluded that this asynchrony is the most consistent and reliable criterion for distinguishing myeloma cells from plasma cells obtained from patients without any sign of multiple myeloma They also found that the degree of asynchrony correlated well with the clinical score they determined for their patients

Our results support those of [illegible] con [illegible] a [illegible] 16[illegible] cells we studied were slightly to markedly asynchronous

With respect to clinical stage versus degree of asynchrony, we have compared the survival time with the degree of asynchrony Except for patient AS, who is still alive and in whom most myeloma cells are only slightly asynchronous we were unable to demonstrate any pronounced relationship

DISCUSSION

This paper describes the ultrastructure of myeloma cells, i e plasma cells from patients with multiple myeloma Consecutive bone marrow specimens were obtained from 13 patients during the clinical course of their disease A total of 65 biopsies were available for study, and from these 1600 sectioned plasma cells were examined

The clinical course of multiple myeloma varies from patient to patient and in most cases is unpredictable Several investigators (1, 3, 5, 16) have attempted to correlate a number of ultrastructural characteristics of myeloma cells with different biochemical and clinical parameters of the patients in order to achieve a reliable prognosis for the disease The present time-related investigation of samples from 11 patients during their survival period is a further attempt to evaluate this possibility

The morphological analysis of the plasma cells from the individual patients was carried out during the course of the disease In addition, it was considered valuable to compare the morphology of plasma cells from the first biopsy of untreated patients with that of cells from biopsies of the same patients during their treatment We can thus confirm earlier described morphological differences between myeloma cells (16, 23, 28) obtained from different patients However, each individual patient presented a characteristic and rather constant »myeloma cell picture«

The size of the myeloma cells varied considerably between individual patients Most of these cells possessed microvilli-like extensions on their surface together with a varying number of pseudopods and it is tempting to speculate whether the pseudopods participate in the movement of the cell (4) or if they represent the morphological basis for the secretion of immunoglobulins, clasmatosis (33) On the other hand, since only one patient (OB) showed a substantial number of myeloma cells with pseudopods in all biopsies, these structures do not seem to be absolutely necessary for the secretion of the immunoglobulins produced by the cells

The main part of the cytoplasm in the myeloma cell is occupied by cisternae of the rough ER Several investigators (1, 23, 28) have grouped the myeloma cells into as many as six different types based on the morphology of the rough ER The results of an attempt to correlate these morphological differences with the class of M component produced have been of limited value In our study, the lamellar type of the rough ER was the most common in the myeloma cells Apart from the patients with Russell bodies (EH, EJ, OB), only one patient (MN) had plasma cells where most of the rough ER was of the globular type This patient had a very short survival time

Annulate lamellae (21) were found in close relation to the cisternae of the rough ER in plasma cells from two patients (MN, ES) Such lamellae have been described previously in myeloma cells (16), but their significance and origin have not been clarified (15)

Circular arrangements of the rough ER with one to three filaments interposed between the cisternae were occasionally found in plasma cells from four of the patients (KC, OB, CB, AS) This type of structure has been described by *Krull et al* (22) in bone marrow plasma cells of a patient who secreted Bence Jones protein of the kappa type Fig 12 in the paper of *Graham & Bernier* (16) shows a longitudinal section of a similar structure in a plasma cell from a patient secreting monoclonal IgG type kappa These structures bear some resemblance to the »ribosome-lamellae-complex« described in hairy cells (20) and myeloma cells (25) In contrast to the last mentioned complexes, those in our study always presented their filaments interposed between two cisternae of the rough ER Neither did we find a regular attachment of ribosomes to the concentric membranes of the rough ER, as described for the hairy cell complex (20) Similar filaments were found between the cylindrical arrangement of cisternae of the rough ER in some plasma cells of a patient with a serum M component of the IgG type lambda (Blom, unpublished work) This M component was shown to possess antistreptolysin O activity (24)

Some mitochondria with an abnormal morphology were observed in the myeloma cells of some patients, but their presence could not be correlated with presence or absence of other characteristic structures However, the mean number of mitochondria in the sectioned plasma cells was very low for the two patients who are still alive This can probably be taken as an indication of the low activity level of these myeloma cells This again could be a sign of a better prognosis but a significant correlation could not be found between the survival time and the mean number of mitochondria in the myeloma cells of the other patients

The ultrastructure of contact zones between plasma cells and dendritic bone marrow macrophages in patients with multiple myeloma has been described previously (7, 8) These contact zones were found in plasma cells in all the biopsies, except in cells with very irregular nuclei where they only occurred occasionally The significance of the contact zones is still unknown However, it has been

hown that their occurrence is not restricted to myeloma cells, as contact zones have been found in plasma cells from patients without a serum M component (Blom, unpublished work) Our earlier hypothesis that the contact zones were most frequent in the bone marrow of patients at the terminal stage of their disease (8) could not be corroborated in this study Another type of contact zones was observed between bone marrow plasma cells and a strip of basement membrane or a lamina densa like material Plasma cells with such contact zones were often seen close to a small vessel A structurally similar intimate contact with small vessels has been described in perivascular plasma cells of brain tissue from patients with multiple sclerosis (26)

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Our results support those of *Graham & Bernier* concerning the nuclear/cytoplasmic asynchrony as a reliable criterion for myeloma cells Most of the 1600 myeloma cells we studied were slightly to markedly asynchronous

With respect to clinical stage versus degree of asynchrony, we have compared the survival time with the degree of [illegible]

[illegible] to demonstrate any pronounced relationship

However, our study has shown that plasma cells with slight asynchrony constitute a significantly greater fraction of the cells in patients under than before treatment

In nine of the patients where the serum contained monoclonal IgG, the response to chemotherapy was associated with a reduction of the concentration of the serum M component as well as of the degree of nuclear/cytoplasmic asynchrony. This observation is in agreement with the findings reported for two of the patients studied by *Graham & Bernier* (16) and is compatible with the conception that cells with a high degree of asynchrony are especially susceptible to chemotherapy

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MORPHOLOGICAL CHANGES AND FATTY ACID COMPOSITION IN HEARTS FROM PIGS FED RAPESEED OIL, FISH OIL, PARTIALLY HYDROGENATED FISH OIL, PARTIALLY HYDROGENATED SOYBEAN OIL AND LARD

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Svaar H Langmark F T Lambertsen G & Opstvedt J Morphological changes and fatty acid composition in hearts from pigs fed rapeseed oil fish oil partially hydrogenated fish oil partially hydrogenated soybean oil and lard Acta path microbiol scand Sect A 88 41–48 1980

Female pigs fed diets in which 42% of the caloric intake came from either rapeseed oil fish oil partially hydrogenated fish oil partially hydrogenated soybean oil or lard were killed after one week, five weeks six months and one year Type of fat or length of feeding did not affect the cardiac content of total fat which was normal in all animals The fatty acid pattern of tissue triglycerides only partly reflected the fatty acid pattern of diets The relative amounts of C22 1 and C20 1 were greatest after six months and levelled off during the following six months The content of C22 1 in cardiac triglycerides never exceeded one fifth of the dietary concentration Microscopic lipidosis was found in some pigs after one week five weeks and six months Minor heart lesions consisting of focal necrosis of muscle cells were found after one week and more frequently after six months and one year No relationship between incidence and severity of the heart lesions and any particular type of fat in the diet could be found

Key words Rapeseed oil fish oil cardiac triglycerides heart lesions pigs

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When moderate to high levels of rapeseed oil are included in experimental diets certain myocardial effects may occur depending on the species employed and the length of the feeding period Most experiments have been carried out with rats but some also with ducklings miniature pigs gerbils and monkeys (1 2 3 6 7 15) In the weanling rat the initial effect is an accumulation of fat in the heart muscle cells which appears after a few hours and reaches a maximum after one week Later it declines gradually in spite of continuous feeding with rapeseed oil The myocardial lipidosis is dose related to the erucic acid content in rapeseed oil when erucic acid constitutes more than 2% (w/w) of the diet (6) When rapeseed oil is given continuously to weanling rats for weeks and months increasing numbers of focal myocardial cell necrosis and cardiac fibrosis are found The severity of the cardiac lesion may be dependent on the dietary level of erucic acid but identical changes have been found in rats receiving rapeseed oil with low erucic acid content (15)

It is of interest to examine whether fish oils containing small amounts of erucic acid and other

long-chain mono unsaturated fatty acids cause the same heart changes as rapeseed oil

In the first scientific report on cardiac effects of rapeseed oil *Roine et al* (13) described the findings in rats and pigs In rats cardiac lesions were found after 2–8 weeks In pigs, the changes were much less pronounced, and similar changes were observed when soybean oil was the fat source Pigs are omnivorous like humans and they are fairly similar to man as regards plasma lipoproteins (10) and coronary circulation (17)

In the present study, domestic pigs were fed fat rich diets containing rapeseed oil fish oil, partially hydrogenated fish oil partially hydrogenated soybean oil or lard for 1, 5, 27 and 52 weeks, and their hearts were examined at each stage for evidence of lipidosis and cardiac lesions and for alterations in fatty acid composition by chemical analysis

MATERIAL AND METHODS

Animals and Experimental design

Female piglets of the Norwegian landrace breed aged between 40 and 80 days (body weight range 10–17 kg) were obtained directly from local breeders A total of 42 piglets came from 8 litters Two piglets were killed before the start of the experiment The remaining 5 piglets from each litter were randomly allocated to the five experimental groups to a total of 8 piglets per group Piglets in each experimental group were kept in two separate pens of four piglets in each Pen temperature and humidity were controlled

Diets

Prior to the experiment the piglets had been nursed by their mothers and in addition received a commercial creep feed given *ad lib* and consisting of 2% dried skim milk 3% herring meal 11% soybean meal 15% maize 25% barley 24% wheat 12% molasses 1 5% sugar 4% grass meal and 2 5% minerals and vitamins

The pigs were fed the experimental diets without an adaptation period The five different diets contained 16% (w/w) from the following fats rapeseed oil (RO) partially hydrogenated capelin oil (HCO) or capelin oil (CO) partially hydrogenated soybean oil (HSO) or lard (L) The composition of the diets was (all figures in % (w/w)) barley 39 solvent extracted soybean meal 26 ground oat hulls 15 sunflower oil 1 5 fat 16 mineral mixture 3 potassium monophosphate 0 5 vitamin mixture 0 45 The mineral mixture consisted of (all figures per kg of the diet) Ca 7 5 g P 1 8 g NaCl 4 73 g Fe 48 mg Mn 60 mg Zn 51 mg Se 0 1 mg I 2 2 mg The vitamin mixture consisted of (all figures per kg of the diet) Vit A 2800 I U Vit D3 400 I U Vit E 100 I U Thiamine HCl 2 6 mg Riboflavin 6 mg Niacin 44 mg Pantothenic acid 26 mg Choline Cl 1 5 mg Vit B12 0 044 mg Pyridoxin Cl 3 mg Ethoxyquine 300 mg DL methionine 400 mg The composition of the diets gives 3330 kcal per kg diet in metabolizable energy of which 42 % comes from fat Protein constituted 17% (w/w) of the diet and the concentrations of lysine and methionine were 0 9% and 0 5% (w/w) respectively

The rapeseed oil was obtained in an unrefined form The unrefined (raw) and partially hydrogenated capelin oils were prosessed from winter caught capelin (Mallotus villosus) under commercial conditions The partially hydrogenated soybean oil was also a commercial sample and like the partially hydrogenated capelin oil was hydrogenated to a melting point of 31–33 °C

Hydrogenated soybean oil was included in the study for its content of trans fatty acids as a control for the hydrogenated fish oils Lard does not contain C22 fatty acids or trans fatty acids and served as a control fat

Table 1 gives the composition of the fatty acids extracted from the feed Batches of feed were prepared at the start of the experiment and again after 150 180 and 240 days Immediately after mixing the diets were stored in sealed polyethylene bags at ambient temperature (0–20 °C) Each bag contained feed for a maximum of 3 days consumption

During the first two weeks the piglets had access to feed twice a day each of one hour duration Later a feeding schedule based on live weight was introduced increasing from 1 kg per pig per day at 35 kg to 3 kg per pig per day at 100 kg and above Individual feed intakes were recorded daily Water was available *ad lib* Live weights were recorded on arrival once a week and prior to killing Two pigs per treatment group were slaughtered after 1 5 27 37 (treatment group CO only) and 52 weeks

Complete autopsies were performed and all organs were weighed after dissection of attached fat Tissue samples were taken from the heart liver kidneys lungs adrenals spleen small and large intestines skeletal muscle (from the left thigh) adipose tissue (from below the right shoulder) and aorta The hearts were cut transversally in 5 mm thick sections from the apex to the base Every second section was fixed for morphological examination the rest was frozen for chemical lipid analysis

Morphological Examination

The hearts were examined by light microscopy for presence of lipidosis and for cardiac lesions Standardized blocks from the left anterior wall were frozen sectioned and stained with Oil Red 0 for demonstration of fat and the findings were graded as follows 0 = negative grade 1 – lipid droplets in less than 25% of the muscle cells grade 2 lipid droplets in 25–50% of muscle cells grade 3 – lipid droplets in 50–75% of muscle cells and grade 4 – lipid droplets in more than 75% of the cells

Three complete transverse sections from each heart were embedded in paraffin and stained with Hematoxylin Phloxine Saffron(14) This method was chosen to make a good distinction between connective tissue and other cardiac structures Cardiac lesions consisted of muscle cell necrosis accumulation of inflammatory cells

TABLE I *Fatty Acid Composition of the Dietary Fats Extracted from the Feed*

Fatty acids	L Lard	HSO Hydrogenated soybean oil	HCO Hydrogenated capelin oil	CO Capelin oil	RO Rapeseed oil
14 0	1 9	0 3	6 8	5 7	–
16 0	20 9	11 5	12 0	9 2	4 2
16 1	3 8	0 4	7 1	6 1	0 1
18 0	14 8	8 1	3 9	1 5	1 4
18 1	40 7	62 1	15 9	13 0	13 1
18 2	12 6	13 2	7 6	6 3	17 4
18 3	1 6	0 9	–	1 5	10 3
18 4	–	–	–	3 6	–
20 0	–	–	3 0	–	–
20 1	1 5	0 5	15 2	14 4	8 3
20 2–3	–	–	2 4	–	–
20 4	0 2	–	–	–	–
20 5	–	–	–	8 0	–
22 0	–	0 5	2 2	–	–
22 1	–	0 5	17 8	18 0	42 2
22 2–4	–	–	3 1	–	–
22 5	–	–	–	1 0	–
22 6	–	–	–	8 7	–
Others	2 0	2 0	3 0	3 0	3 0
Ratio of unsaturated/saturated fatty acids	1 6	3 8	2 5	4 9	16 3

and areas of connective scar tissue The cardiac findings were graded as follows grade 1 ~ one focal lesion consisting of necrotic muscle cells and inflammatory cells or a small focal scar grade 2 = two or three focal lesions of grade 1 type grade 3 = more than three focal lesions or larger confluenting lesions covering more than one high power microscopical field Frozen sections from the liver adrenals skeletal muscle kidneys and spleen were stained for fat with Oil Red 0 Sections from all organs were stained with Hematoxylin Eosin All specimens were studied independently by two pathologists without prior knowledge of the dietary group

Method of Analysis

Chemical analysis of feed ingredients was done by conventional methods Each diet was sampled and a composite was stored at −20 °C in a sealed box pending analysis The samples were extracted with chloroform/methanol to give total lipids from which fatty acid methyl esters were prepared and analysed by gas liquid chromatography

Pieces of 5 g were taken from frozen samples of each heart liver and skeletal muscle and pooled for each experimental group thus 10 g samples were processed per organ per group

Lipids were extracted from the ground samples with chloroform/methanol (7 3) The lipid extracts were

...octane to give a 5% solution for gas chromatography These samples were stored in closed vials at −20 °C until analysed ...

were

peak

half

estimated and the peaks were identified by standard runs of saturated acids and by semilog plots ...

...

ac

14

ch

ac ... and medium unsaturated were given as 20 2–3 and 22 2–4

long-chain mono unsaturated fatty acids cause the same heart changes as rapeseed oil

In the first scientific report on cardiac effects of rapeseed oil, *Rotne et al* (13) described the findings in rats and pigs In rats, cardiac lesions were found after 2–8 weeks In pigs, the changes were much less pronounced, and similar changes were observed when soybean oil was the fat source Pigs are omnivorous like humans and they are fairly similar to man as regards plasma lipoproteins (10) and coronary circulation (17)

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TABLE 3 *Fatty acid composition (%) of the heart triglycerides of pigs on a 42 cal% fat diet*

Fatty acids	at start	L				HSO				HCO				CO				RO			
		1	5 (weeks)	27	52	1	5 (weeks)	27	52	1	5 (weeks)	27	52	1	5 (weeks)	27	37	1	5 (weeks)	27	52
14 0	0 6	1 9	0 5	1 9	0 7	1 5	1 4	0 9	0 5	2 2	1 2	3 8	1 9	5 4	1 8	3 5	2 0	1 0	0 4	1 1	0 4
15–	–	1 2	7 3	0 9	4 9	1 8	1 2	–	2 5	0 5	4 8	0 6	3 2	1 9	4 1	1 2	6 5	1 8	2 7	–	2 3
16 0	14 5	18 7	20 3	19 3	16 0	19 9	15 6	14 8	12 6	20 0	11 2	16 3	15 6	13 8	14 6	17 8	15 8	16 6	11 8	12 7	13 9
16 1	1 6	6 0	1 8	4 4	2 0	6 9	4 6	3 5	1 8	8 4	3 8	8 6	5 0	4 2	4 1	4 9	3 8	5 0	1 6	2 6	0 9
17–	–	–	2 0	–	2 7	–	–	–	1 6	–	2 3	–	1 4	–	1 0	–	3 8	–	–	–	0 8
18 0	15 0	12 5	19 5	9 6	13 1	10 7	10 3	9 0	11 9	8 5	12 7	6 2	11 9	12 5	12 2	7 7	15 3	11 5	11 3	4 9	12 1
18 1	15 6	29 6	25 0	34 9	31 3	29 1	38 9	40 3	36 7	28 6	14 8	28 1	19 3	16 4	21 6	23 0	23 2	22 9	14 4	26 1	19 2
18 2	26 8	15 8	16 8	16 4	14 6	16 7	18 4	20 1	19 8	13 4	28 4	15 5	21 3	14 3	12 8	10 6	11 4	17 2	28 4	21 0	26 0
18 3	0 5	0 7	0 3	0 7	0 6	0 7	1 1	1 6	0 9	1 1	1 0	2 5	0 9	0 7	1 0	1 1	0 8	2 3	4 2	5 7	3 4
20 1	0 7	0 7	0 7	1 1	0 9	0 7	0 9	1 2	0 5	3 5	3 2	9 0	5 6	4 2	3 2	10 3	6 7	2 4	4 4	8 5	5 4
20 2–3	0 6	1 4	0 7	0 9	0 4	0 9	0 1	–	0 3	0 8	0 9	1 5	1 1	0 6	0 4	–	0 8	1 2	1 1	0 8	1 4
20 4	13 0	6 9	1 1	5 0	8 3	6 4	3 6	3 6	6 9	2 6	9 1	1 2	7 4	10 5	4 8	1 1	3 8	6 9	4 6	1 7	4 0
20 5	2 4	0 4	–	0 4	0 5	0 4	0 3	0 5	0 5	2 0	0 8	0 8	0 4	5 5	9 4	6 3	0 7	1 2	0 9	–	0 2
22 1	–	–	0 5	–	–	–	–	0 3	–	2 0	2 2	2 4	1 2	3 8	1 4	4 2	1 7	5 4	9 8	10 5	6 4
22 2–4	0 8	0 3	0 5	0 5	0 3	0 3	0 2	0 4	0 3	0 5	0 4	–	0 5	1 1	0 8	0 9	0 7	0 7	1 0	0 8	0 6
22 5	1 8	0 4	–	0 6	0 3	0 4	0 2	0 4	0 1	0 9	0 2	0 6	0 2	0 5	0 8	1 2	–	0 4	0 4	0 5	–
22 6	3 1	0 5	–	0 4	0 4	0 6	0 2	0 3	0 1	2 0	–	–	0 1	1 6	3 0	3 2	–	0 5	–	–	–

Pooled data from 2 pigs per sampling

TABLE 2 *Number of Pigs with Microscopical Lipidosis and Heart Muscle Necrosis*

Dietary fat	Lipidosis				Heart muscle necrosis			
	1	5 (weeks)	27	52	1	5 (weeks)	27	52
Lard	0	0	0	0	0	0	1	1
Hydrogenated soybean oil	0	0	0	0	1	0	1*	1
Hydrogenated capelin oil	1	0	1	0	0	0	1	1
Raw capelin oil	0	0	0	–	0	0	0	–
Rapeseed oil	1	2*	1*	0	1	0	1	2

2 pigs per sampling and group All positive hearts had grade 1 lipidosis or grade 1 heart muscle necrosis except those marked with a * * = one heart with grade 2 findings

RESULTS

General Conditions and Growth

In general all pigs showed normal appearance throughout the experimental period and had adequate growth rate and feed consumption In the initial part of the experiment a few episodes of diarrhoea occurred At the later stages some cases of limb weakness were seen None of these conditions are unusual among pigs and there was no relationship between the conditions and the diets After 37 weeks the remaining two pigs in the CO group suffered from mechanical bone fractures and had to be killed earlier than planned All diets were readily consumed although the pigs fed RO and HSO had a slightly lower feed consumption compared to the other pigs during the first 2 weeks when they had free access to the feeds The pigs fed rapeseed oil grew at a lower rate compared to the other groups ($p < 0.01$) the first half year consuming 13% more feed per kg live weight gain than the other groups In the last half year no differences in growth rate were seen The weight range after one year was 178–257 kg



Figure 1 Grade 1 cardiac lesion with necrosis of individual muscle cells and infiltration of leucocytes and histiocytes HPS 400 ×

Morphological Results

Heart No macroscopic changes were found at autopsy in pigs killed after 1 or 5 weeks The amount of epicardial fat was markedly increased after 27 and 52 (or 37) weeks in all groups

Table 2 gives the result of the histological examination Lipidosis was found in individual heart muscle cells in all parts of the heart walls but more frequently in the sub epicardial areas The lipid droplets were small also in grade 2 hearts In the hearts taken at one year no lipidosis was found Cardiac lesions were all small and focal and consisted of individual muscle cell necrosis with focal accumulation of leucocytes and histiocytes (Fig 1) Some muscle cells with vacuolization and disintegration of myofibrils could be found around the lesions Large groups of mature fat cells surrounded by atrophic muscle fibers were seen in all parts of the heart walls This lipomatosis appeared more frequently in the older pigs and was found in all dietary groups

Other organs In the aorta small foci of fatty streaks were found in most

TABLE 3 *Fatty acid composition (%) of the heart triglycerides of pigs on a 42 cal% fat diet*

Fatty acids	at start	L				HSO				HCO				CO				RO			
		1	5 (weeks)	21	52	1	5 (weeks)	27	52	1	5 (weeks)	27	52	1	5 (weeks)	27	37	1	5 (weeks)	27	52
14 0	0 6	1 9	0 5	1 9	0 7	1 5	1 4	0 9	0 5	2 2	1 2	3 8	1 9	5 4	1 8	3 5	2 0	1 0	0 4	1 1	0 4
15 –	–	1 2	7 3	0 9	4 9	1 8	1 2	–	2 5	0 5	4 8	0 6	3 2	1 9	4 1	1 2	6 5	1 8	2 7	–	2 3
16 0	14 5	18 7	20 3	19 3	16 0	19 9	15 6	14 8	12 6	20 0	11 2	16 3	15 6	13 8	14 6	17 8	15 8	16 6	11 8	12 7	13 9
16 1	1 6	6 0	1 8	4 4	2 0	6 9	4 6	3 5	1 8	8 4	3 8	8 6	5 0	4 2	4 1	4 9	3 8	5 0	1 6	2 6	0 9
17 –	–	–	2 0	–	2 7	–	–	–	1 6	–	2 3	–	1 4	–	1 0	–	3 8	–	–	–	0 8
18 0	15 0	12 5	19 5	9 6	13 1	10 7	10 3	9 0	11 9	8 5	12 7	6 2	11 9	12 5	12 2	7 7	15 3	11 5	11 3	4 9	12 1
18 1	15 6	29 6	25 0	34 9	31 3	29 1	38 9	40 3	36 7	28 6	14 8	28 1	19 3	16 4	21 6	23 0	23 2	22 9	14 4	26 1	19 2
18 2	26 8	15 8	16 8	16 4	14 6	16 7	18 4	20 1	19 8	13 4	28 4	15 5	21 3	14 3	12 8	10 6	11 4	17 2	28 4	21 0	26 0
18 3	0 5	0 7	0 3	0 7	0 6	0 7	1 1	1 6	0 9	1 1	1 0	2 5	0 9	0 7	1 0	1 1	0 8	2 3	4 2	5 7	3 4
20 1	0 7	0 7	0 7	1 1	0 9	0 7	0 9	1 2	0 5	3 5	3 2	9 0	5 6	4 2	3 2	10 3	6 7	2 4	4 4	8 5	5 4
20 2–3	0 6	1 4	0 7	0 9	0 4	0 9	0 1	–	0 3	0 8	0 9	1 5	1 1	0 6	0 4	–	0 8	1 2	1 1	0 8	1 4
20 4	13 0	6 9	1 1	5 0	8 3	6 4	3 6	3 6	6 9	2 6	9 1	1 2	7 4	10 5	4 8	1 1	3 8	6 9	4 6	1 7	4 0
20 5	2 4	0 4	–	0 4	0 5	0 4	0 3	0 5	0 5	2 0	0 8	0 8	0 4	5 5	9 4	6 3	0 7	1 2	0 9	–	0 2
22 1	–	–	0 5	–	–	–	–	0 3	–	2 0	2 2	2 4	1 2	3 8	1 4	4 2	1 7	5 4	9 8	10 5	6 4
22 2–4	0 8	0 3	0 5	0 5	0 3	0 3	0 2	0 4	0 3	0 5	0 4	–	0 5	1 1	0 8	0 9	0 7	0 7	1 0	0 8	0 6
22 5	1 8	0 4	–	0 6	0 3	0 4	0 2	0 4	0 1	0 9	0 2	0 6	0 2	0 5	0 8	1 2	–	0 4	0 4	0 5	–
22 6	3 1	0 5	–	0 4	0 4	0 6	0 2	0 3	0 1	2 0	–	–	0 1	1 6	3 0	3 2	–	0 5	–	–	–

Pooled data from 2 pigs per sampling

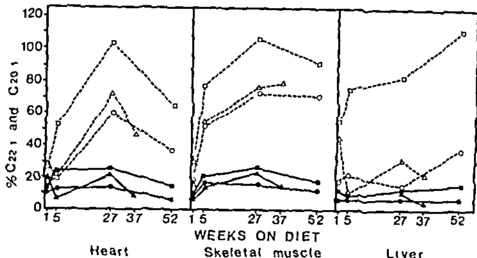


Figure 2 The relative amounts of C22 1 and C20 1 in the heart, liver and skeletal muscle The amount of C22 1 and C20 1 in the diets is given as 100% ——— = C22 1 ---------- = C20 1 □■ = Rapeseed oil △▲ = Raw capelin oil ○● = Partially hydrogenated capelin oil

weeks Fibrous or advanced lesions were not seen No relation was found between appearance or number of fatty streaks and dietary fats In skeletal muscle, trace amounts of lipid droplets were seen in all groups No significant morphological changes were found in the lungs, kidneys, livers, spleens, adrenals, intestines or the adipose tissue, and the relative organ weights were similar in all groups

Lipid in the Hearts

The amount of total lipids extracted varied somewhat at different periods without any tendency to increased accumulation in any particular diet The average amount extracted from all hearts was 48 1 mg/g heart

Fatty Acid Patterns in Hearts

Table 3 gives the fatty acid composition in the heart triglycerides as weight percentages

The levels of C22 1 and C20 1 were of primary interest in this study Both C22 1 and C20 1 increased in the hearts, but the peaks were not reached before 27 weeks on RO or fish oils Fig 2 shows the relative amounts of these acids in the hearts, livers and thigh muscles at different sampling times The values are the concentration of C22 1 and C20 1 in the heart triglycerides as a percentage of the concentration of C22 1 and C20 1 in the respective dietary fats Much higher levels of C20 1 than of C22 1 were evident in all tissues The C22 1 was never found in the tissue lipids in concentrations above 25% of the dietary level The relative concentrations of both monoenes were higher in the tissue triglycerides from the rapeseed oil group than from the two fish oil groups Heart muscle and skeletal muscle tissue had similar levels of the two acids during the experiment The concentration of 22 1 and C20 1 increased through the first half year and showed lower values at the one year sampling aompared to the value at six months

The essential fatty acids, C18 2omega6 (linoleic acid) and C20 4omega6 (arachidonic acid), were found as major acids in most of the samples of the heart, liver and skeletal muscle

C18 1 was higher in the hearts in the fish oil and RO groups than in the other groups The average tissue levels (all sampling periods together) were 7% higher than the dietary levels of C18 1 in these groups C18 1 was the major acid in HSO and L, and although the tissue levels of this acid were high they were lower than the dietary levels in HSO (~26%) and L (~10%) Similar results were found in the liver and thigh muscle samples Detailed analytic data of the fatty acid patterns in skeletal muscle and liver may be obtained on request (G L)

DISCUSSION

The pigs fed rapeseed oil had a lower growth rate and a poorer efficiency of feed conversion than pigs fed the other fats, which is in line with previous findings (5) The digestibility however was not determined and it is therefore not possible to conclude whether differences in absorption or in metabolism of the absorbed fatty acids account for this

There was no increase in total fat content in the hearts in any dietary group But lipidosis of grade 1–2 (slight to moderate) was found in pigs fed rapeseed oil or hydrogenated fish oil The lipidosis

probably reflects an altered distribution of the lipids in the heart muscle cells with formation of larger lipid droplets than normally present

The type of rapeseed oil used caused a grade 4 lipidosis in rats after one week, and the findings indicate considerable species differences between pigs and rats (12)

The fatty acid composition of the triglycerides of the hearts livers and skeletal muscles of the pigs partly reflected the fatty acid composition of the dietary fats Heart triglycerides from pigs fed fish oils and rapeseed oil contained on an average 2 4% and 8 0% of C22 1 respectively which is about 1/5–1/6 of the concentrations in the respective dietary fats On rapeseed oil the content of C22 1 in the heart triglycerides after 5 and 27 weeks of feeding was about twice that found after 1 and 52 weeks This protracted peaking in C22 1 content is in agreement with other observations in pigs (7 16) Further the maximum levels of C22 1 found in triglycerides of the heart and other tissues in the pigs were much lower than those found in rats after one week of feeding on rapeseed oil (12) On fish oil the content of C22 1 in heart triglycerides showed no clear-cut relation to the time of feeding

In general the concentrations of C22 1 and C20 1 in tissue triglycerides as a percentage of the corresponding dietary lipids were higher on the rapeseed oil than on the fish oils This result may arise from differences in double bond position in the monoene fatty acids in rapeseed oil and fish oil In rapeseed oil 100% of the C22 1 is erucic acid (C22 1omega9) while hydrogenated fish oil contains a wide range of positional and geometrical isomeric forms of C22 1 fatty acids of which erucic acid constitutes approximately 1/10 (11) The concen tration in tissue triglycerides as a percentage of the dietary concentration was higher for C20 1 than for C22 1 It is possible that the absorption rates of C22 1 and C20 1 are different in the pig but this does not seem very likely The data probably indicate that C22 1 is more readily metabolized to its lower homologues in the pig than in other animals studied The relative high content of C18 1 in tissue triglycerides compared with that of the dietary fat in pigs fed rapeseed oil and fish oils may also support this assumption

It is fairly well established that the continuous feeding of rapeseed oil rich in erucic acid like that used in this study causes extensive and numerous heart muscle lesions in rats when fed for periods longer than 6 months In this study on pigs definite cardiac lesions were found in all dietary groups and more frequently after 27 and 52 weeks than after shorter feeding periods The appearances of the lesions were identical with those found in rats fed similar fats but the relatively low incidence and mildness of the lesions contrast with the findings in rat experiments (3, 8)

It is noteworthy that identical lesions were found in the lard and soybean oil groups (i e the controls) as those found in the rapeseed oil groups *Roine et al* (13) described »myocarditis like« lesions in pigs fed 28% of calories from rapeseed oil for 2 months, but found the same amount and severity of lesions in pigs fed soybean oil as control fat Similar findings have been reported by *Friend et al* (9) and *Aherne et al* (4) The pathogenesis of these lesions remains unknown but at the present time it seems more probable that they are caused by factors other than the consumption of C22 1 fatty acids by the pigs

In the present study the dietary concentrations of C22 1 fatty acids were 7 4% and 3 2% in the rapeseed oil and fish oil diets respectively and only a mild degree of lipidosis was found The data from this study as well as other studies on pigs cited above do not suggest that dietary C22 1 and C20 1 are cardiotoxic in pigs

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CARCINOEMBRYONIC ANTIGEN IN FETAL TISSUES AND IN MATERNAL SERUM

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Lindgren J Carcinoembryonic antigen in fetal tissues and in maternal serum Acta path microbiol scand Sect A *88* 49-53 1980

The occurrence of CEA was studied by radioimmunoassay and the triple bridge immunoperoxidase technique in tissues of human fetuses and by radioimmunoassay in fetal and maternal serum The antiserum to CEA was extensively adsorbed to eliminate cross reactions with CEA related antigens After the first trimester of pregnancy CEA was found throughout the gastrointestinal tract by both techniques whereas the other fetal tissues and fetal serum did not contain detectable amounts of CEA The CEA level of all 69 sera of pregnant women was below 2 5 ng/ml The excretory nature of CEA was suggested by the localization of CEA in the luminal border of the alimentary tract and by the finding that the CEA concentration in the content of fetal gastrointestinal canal was higher than in the surrounding tissue Gel filtration on Sepharose 4B showed that molecular weight of CEA immunoreactive material of the fetal gut was similar to that of CEA purified from colon cancer but a minor component with a higher molecular weight was eluted in the void volume When tested in radioimmunoassay the CEA immunoreactive material in both peaks gave an inhibition curve parallel to that of purified CEA

Key works Carcinoembryonic antigen fetal tissue maternal serum

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In this report the expression of CEA in fetal organs was studied both immunohistochemically and by radioimmunoassay The presence of CEA in maternal serum at different stages of gestation was also determined Molecular weight and immunological properties of fetal CEA reactive material were studied and compared with the CEA purified from liver metastases of human colon cancer

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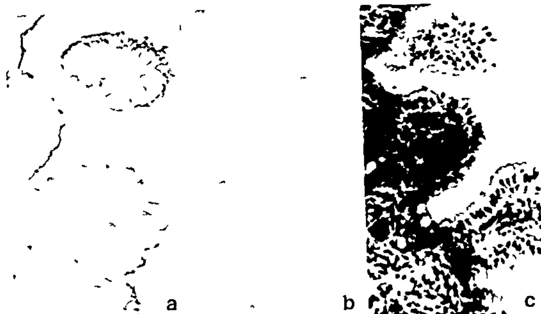


Fig 1 Demonstration of CEA on the secretory border of the fetal stomach a Immunoperoxidase staining with antiserum to CEA b Staining with antiserum to CEA adsorbed with CEA c Hematoxylin-eosin

tissues did not contain detectable amounts of CEA (Table 1) In fetus No 4 the contents of the intestine at different sites were studied separately The highest CEA concentrations up to 283 ng/mg protein were found in the luminal contents of the jejunum and colon (Table 2)

CEA in fetal and maternal serum No demonstrable CEA (< 1 0 ng/ml) was found in any of the seven serum samples from fetuses between the 21st and 32nd weeks of gestation In one out of the 69 sera of pregnant women between the 8th and 42nd weeks the serum CEA level was 1 4 all the other levels being less than 1 0 ng/ml

Immunoperoxidase staining of CEA After the 12th week of gestation a positive staining reaction was seen at the luminal border of all parts of the gastrointestinal tract from the esophagus to the rectum No cytoplasmic staining was seen and the goblet cells were consistently negative (Fig 1)

Molecular weight of CEA immunoreactive material The main peak of CEA reactivity eluted in the same volume as purified CEA when chromatographed on a Sepharose 4 B column A minor peak of CEA immunoreactivity appeared in the void volume (Fig 2)

Immunological characterization of the CEA reactive material A precipitation line of complete identity was seen in immunodiffusion when concentested with purified CEA against the absorbed antiserum to CEA Gel filtration fractions containing high CEA concentrations gave inhibition curves parallel to that of purified CEA in radioimmunoassay (Fig 3)

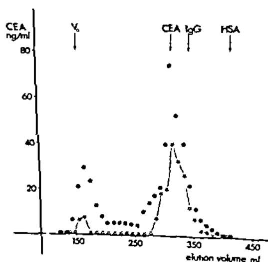


Fig 2 Gel f[illegible]

MATERIALS AND METHODS

Antigens and antisera The purification of CEA and the generation and characterization of antisera have been described (*Hammarström et al 1975, Lindgren et al* 1979) CEA was purified from liver metastases of colon *carcinoma using ion exchange* chromatography, gel filtration and affinity chromatography The antiserum to CEA was raised in rabbits with purified CEA To eliminate reactivity with cross-reacting antigens the serum was adsorbed with A, B, and O human red blood cells, normal human *serum coupled to CNBr-activated* Sepharose 4B (Pharmacia, Uppsala, Sweden) and with *Sepharose-coupled* 1 M perchloric acid (PCA)-extracts of normal human spleen (NCA), meconium (NCA 2), and gastric juice (CELIA)

Fetal tissue specimens for radioimmunoassay Tissue samples including the rectum, colon, small intestine, stomach, esophagus, *pancreas, liver, spleen* lung, kidney, thymus muscle, brain, and adrenal gland were *dissected from four* apparently normal fetuses legally aborted at the 10th, 13th, 18th, and 20th weeks of pregnancy (numbers 1, 2, 3, and 4, respectively) Fetus No 5, born at the 31st week of gestation, presented with an adrenogenital syndrome and died at the age of one week The *specimens were taken into phosphate buffered* saline, pH 7 4 (PBS) After freezing and thawing the *specimens were sonicated at* 70 W for two minutes, centrifuged at 1000 × g for 15 minutes and the supernatants collected for CEA and protein determinations

Serum samples Serum samples were taken by cardiac puncture from seven *stillborn fetuses between the 21st* and 32nd weeks of gestation 69 maternal serum samples *were obtained from pregnancies between the* 8th and 42nd weeks All sera were inactivated at 56 °C for 30 minutes and stored at −20 °C until tested

Radioimmunoassay for CEA Double antibody assay was used as described (*Rutanen et al* 1978) Supernatants from tissue sonicates were diluted 1 2 with *normal* human serum and serum samples 1 2 with PBS Greater *dilutions were done with 1 2 diluted normal human* serum The sensitivity of the assay was 1 0 ng/ml

Determination of protein The protein content of tissue sonicates was estimated according to *Lowry et al* (1951)

Immunohistochemical staining of CEA Tissue specimens of the rectum colon small intestine stomach esophagus pancreas liver spleen lung kidney, thymus muscle brain and adrenal gland were taken from eight *apparently normal legally aborted fetuses between the 12th and 18th weeks of pregnancy Tissue samples were* fixed with formalin and embedded into paraffin The *triple-bridge immunoperoxidase method was applied as* described (*Lindgren et al* 1979) Briefly after deparaffinization and rehydration the tissue sections were incubated sequentially with the adsorbed antiserum to CEA sheep anti-rabbit IgG rabbit anti horseradish peroxidase peroxidase and finally the substrate To minimize nonspecific staining the sections were first treated with cold methanol containing 0 5 % hydrogen peroxide and with 2% normal sheep serum between each step Two *adjacent control* sections were stained either with the same antiserum completely adsorbed with purified CEA or with hematoxylin-eosin A positive reaction for CEA was a brown colour which appeared in the test slide but not in the adjacent *control section The staining* characteristics of the antiserum have been described (*Lindgren et al* 1979)

Gel filtration Colonic and esophageal extracts of fetus No 4 were chromatographed on a Sepharose 4 B column (82 × 2 5 cm, Pharmacia) equilibrated with 0 05 M tris HCl buffer, pH 7 5 The column was calibrated with human IgG, albumin (*Kabi, Stockholm, Sweden*) and 125I-labelled purified CEA The CEA concentration of *fractions was measured by radioimmunoassay* as described above For radioimmunoassay the fractions were 5–10 times concentrated by lyophilization

RESULTS

CEA concentration in fetal tissue CEA was found by radioimmunoassay throughout the fetal gastrointestinal tract in all fetuses studied, whereas other

TABLE 1 *The CEA Content of Fetal Tissues ng/mg Protein*

No of fetus	1	2	3	4	5
Age*	10	13	18	20	31
Esophagus		1 1	21 0	47 5	1 4
Ventricle		1 6	14 3	3 7	0 2
Duodenum	5 0**	0 8	2 4	1 8	0 6
Jejunum		2 1	20 0	23 7	4 6
Colon		2 5	8 3	1 2	17 0
Rectum		10 5	NT***	12 5	19 2
Other tissues****	<0 5	<0 5	<0 5	<0 5	<0 5

* weeks of gestation
** tested altogether
*** not tested
**** pancreas liver spleen lung kidney thymus muscle brain and adrenal gland

TABLE 2 *CEA Concentration in the Luminal Contents and the Corresponding Tissue at Different Sites of the Fetal Gastrointestinal Tract The Values are ng/mg Protein*

	Tissue	Luminal content
Esophagus	47 5	NT*
Ventricle	3 7	85
Duodenum	1 8	16 3
Jejunum	23 7	240
Colon	1 2	283
Rectum	12 5	45 5

* not tested

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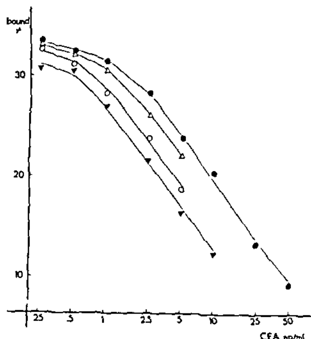


Fig 3 *Inhibition of the gel filtration peaks presented in figure 2 in the radioimmunoassay for CEA* ●——● purified CEA ▼——▼ fetal colonic CEA main peak △——△ *fetal colonic* CEA *void* peak ○——○ fetal esophageal CEA main peak

DISCUSSION

CEA is a normal constituent of the fetal gastrointestinal tract after the first trimester of pregnancy Like in adult tissues (*Go et al* 1975 *Isaacson & Judd* 1977 *Martin & Devant* 1973) CEA is found throughout the fetal gastrointestinal tract from the esophagus to the rectum However high amounts of CEA were found in the esophagus and the jejunum during mid pregnancy whereas the colon and the rectum became more dominant sites of CEA synthesis during the latter half of gestation

The immunological properties molecular weight and the immunohistochemical staining pattern suggest that the fetal CEA reactive material is closely related if not identical to the CEA from colon cancer and different from the known cross reacting substances

Gold & Freedman (1965) and *Khoo et al* (1973) reported the occurrence of CEA like activity in the fetal colon pancreas and liver This was not confirmed in the present study and the discrepancy may be due to differences in the specificities of the antisera used

The excretory nature of CEA (*Go et al* 1975) is suggested by the fact that the CEA concentration of the intestinal contents w is higher than that of the corresponding tissues Therefore it s not surprising that the CEA level in fetal and maternal serum is negligible

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This work was supported by a grant from *The Finnish Cultural Foundation* I thank Ms s *S Kuisma* and *H Kurppa* for excellent technical assistance and Drs *M Seppala* and *P Sipponen* for discussions

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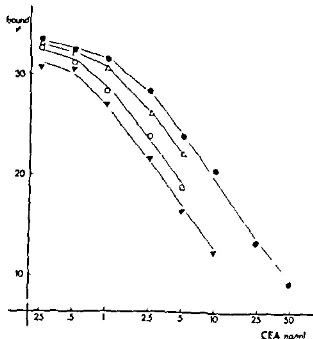


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CONGOPHILIC SUBSTANCE WITH GREEN DICHROISM IN HIP JOINTS IN AUTOPSY MATERIAL

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Ladefoged C & Christensen H E Congophilic substance with green dichroism in hip joints in autopsy material Acta path microbiol scand Sect A 88 55–58 1980

Cartilage and fibrous capsular tissue from the hip joints of 30 unselected autopsies have been examined for the presence of congophilic substance with green dichroism (CS GD) Ninety three per cent of the cases had CS GD in the joint cartilage and 43 per cent contained amyloid deposits in the capsule The relation of these substances to age and the relation of CS GD to the upper and lower parts of the femoral head cartilage have been studied as well as the inter relationship between the presence of CS GD in the cartilage and amyloid deposits in the capsule within each joint

Key words Hip joints congophilic substance autopsy material

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We have previously reported upon the presence of amyloid substance in the fibrous capsule of the hip joint in approximately one third of patients subjected to plastic reconstruction for arthrosis coxae (*Christensen & Sørensen* 1972) A rather constant association between pyrophosphate crystal line arthritis and amyloid deposition has been reported (*Teglbjærg et al* 1979)

The aim of the present study was to determine the number of the above mentioned amyloid deposits in the fibrous capsule exceeding that expected in an unselected autopsy series Further to elucidate the frequency of articular cartilage of the hip joint being the site of CS-GD deposits

MATERIAL AND METHODS

Patient Material

The patient material comprises 30 routine autopsies carried out at a County Hospital serving as a University Hospital Post mortem examination is performed in approximately 90 per cent of the deaths in this hospital The present 30 autopsies were randomly selected among the 223 routine autopsies performed in January and February of 1978 No cases of generalized amyloidosis rheumatoid arthritis tuberculosis or myelomatosis were found in the material

The distribution according to age can be seen from Table 1 The mean age for the whole material was 72 7 years (range 42–97 years) The mean age for the 13 women was 73 3 years (range 54–86 years) and that for the 17 men 72 3 years (range 42–97 years) Hip joint articular cartilage from the upper as well as the lower part of the femoral head was taken for histological examination as were pieces of the joint capsule

The material for histological examination was only taken from the right side in the first 10 autopsies but from both sides in the last 20

Histological Technique

The tissue samples were prepared using routine methods Sections were stained with hematoxylin-eosin and the alkaline Congo red method described by *Puchtler et al* [illegible]

sen [illegible] slight = + moderate = ++ and pronounced = +++

RESULTS

The articular cartilage of the hip joint contained CS-GD in 28 of the 30 autopsies i e in 93 per cent of

TABLE 1 *CS DG in Cartilage (Cart) and Amyloid Deposition in Capsule (Caps) in Different Age Groups*

Age (years)	Grade of deposits									
	0		+		++		+++		Total	
	cart	caps	cart	caps	cart	caps	cart	caps	cart	caps
41 – 50		1			1				1	1
51 – 60	1	3			2				3	3
61 – 70	1	7	4	1	4	1			9	9
71 – 80		3	2	2	4	1	3	3	9	9
81 – 90		2	1		2	2	3	2	6	6
91 – 100		1		1	1		1		2	2
Total	2	17	7	4	14	4	7	5	30	30

TABLE 2 *CS GD in Articular Cartilage at the Upper and Lower Parts of the Femoral Head (Right + Left Side)*

	0	+	++	+++	Total
Upper pole	7 (14)	21 (42)	17 (34)	5 (10)	50
Lower pole	5 (10)	23 (46)	17 (34)	5 (10)	50
Total	12	44	34	10	100

The figures in parentheses are per cent

the cases The deposits were mostly seen as a narrow rim just below the outer limit of the articular cartilage (Fig 1 and 2) Single chondrocytes and small lacunas of atrophic chondrocytes often surrounded by a zone of the material (Fig 3 and 4) were another characteristic feature In some cases it was seen as round or irregular masses in the outer zone of the articular cartilage At times these showed confluence between the superficial rim (Fig 1 and 2) and the congophilia surrounded chondrocytes (Fig 3 and 4) This phenomenon is illustrated in Fig 5

Amyloid deposits were found in the fibrous joint capsule in 13 of the 30 cases i e in 43 per cent of the autopsies often bilaterally (Fig 6)

Relation to age The CS-GD in the cartilage as well as the amyloid deposits in the capsules were larger the higher the age of the patient This is shown in Table 1 There is a significant age relation with Spearman s Rho test (0 001 < P < 0 01) in both [illegible]

TABLE 3 *CS GD in Articular Cartilage Versus Amyloid Substance in Capsule (Right + Left Side)*

Cartilage	Capsule amyloid pos	Capsule amyloid neg	Total
CS-GD pos	17	29	46
CS-GD neg	0	4	4
Total	17	33	50

The relationship between the presence of CS GD in articular cartilage and amyloid deposits in the capsule is illustrated in Table 3

Notice that when amyloid was found in the capsule cartilage always contained CS-GD

DISCUSSION

The frequency of amyloid positive fibrous capsules in a previous work (*Christensen & Sørensen* 1972) was 37 per cent This figure is a little lower than the



Fig 1



Fig 2



Fig 3



Fig 4

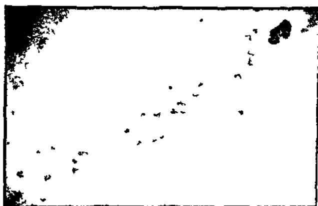


Fig 5



Fig 6

Fig 1 Congophilic deposits seen as a narrow rim just below the surface of the articular cartilage (Alkaline Congo red × 125)

Fig 2 The same area as in Fig 1 showing green dichroism using polarized light (Alkakine Congo red × 125)

Fig 3 Lacunas and single chondrocytes surrounded by a zone of congophilic material (Alkaline Congo red × 350)

Fig 4 The same area as in Fig 3 showing green dichroism using polarized light (Alkaline Congo red × 350)

Fig 5 Congophilic deposits as round or irregular masses in the outer zone of the articular cartilage (Alkaline Congo red × 250)

Fig 6 Amyloid deposits in the fibrous joint capsule (Alkaline Congo red × 250)

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BRIEF REPORT

BREAST CARCINOMA PROGRESSION OF TUBULAR CARCINOMA AND A NEW CLASSIFICATION

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Linell F & Ljungberg O Breast carcinoma Progression of tubular carcinoma and a new classification Acta path microbiol scand Sect A 88 59–60 1980

On histologic and statistic basis a new classification of ductal breast carcinoma is proposed One group consists of five types representing different stages in a continuous progression from tubular carcinoma to pure »ductal« type Another group is comprised by invasive »comedo« carcinoma which is derived from intraductal carcinoma The development of tubular carcinoma from sclerosing lesions is stressed

Key words Breast carcinoma classification tubular carcinoma sclerosing lesions

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The most common classification of breast carcinoma was developed by *Foote & Stewart* (6) in the 1940 s In most materials of invasive carcinoma the infiltrating lobular carcinoma comprises approximately 10 per cent The remaining 90 per cent represent ductal carcinomas of which 65–80 per cent are not otherwise specified (N O S) *Fisher et al* (4) The rare tubular ductal carcinoma occurred in about 1 per cent in the large material of *Fisher et al* (4) based on 1000 cases Also in *Rosen* s (12) material from Memorial Hospital (941 cases from 1976–1978) tubular carcinoma comprised only one per cent

We have studied all breast carcinomas from the city of Malmo during the period 1976–1978 The material consists of 590 cases Fifty seven of these were non invasive Of the remaining 533 invasive carcinomas 35 were not classified for various reasons (inadequate amount of tissue or derangement of histologic structure due to preoperative radiation) The study thus comprises 498 cases of invasive carcinoma We have studied them personally macro- and microscopically and the histologic examination has been extensive Owing to mammographic screening of a large proportion of the population of Malmo during 1977–1978 a relatively large number of small tumours were received for diagnosis

The classification and relative frequency of the carcinomas is given in Table 1

TABLE 1

Tubular carcinoma	21 %
Duct carcinoma N O S	56 2%
Medullary carcinoma with lymphoid stromal infiltration	5 6%
Mucinous carcinoma	4 2%
Lobular infiltrating carcinoma	12 9%

Patchefsky et al (11) in a mammographic screening study found 9 per cent tubular carcinomas A high frequency of tubular carcinoma in screened material is also reported by *Andersson et al* (1)

During this study ample histologic evidence was obtained (*Linell et al* (10)) that tubular carc[illegible]

The high frequency of tubular carcinoma in our series [illegible] be ascribed to the fact that a large

frequency found in the present publication, which was 43 per cent A possible explanation of the difference is that the patients in Christensen and Sørensen's material had a lower mean age than the autopsy material in the present study (63 1 against 72 7 years)

Amyloid substance in the surface layers of articular cartilage was briefly recorded by *Gamarski and Netto* (1959) in a case of primary amyloidosis *Bywaters and Dorling* (1970) made the same findings in two cases exhibiting primary amyloido sis and multiple myeloma respectively These are the only reports of the presence of amyloid substance in articular cartilage

It is not surprising that amyloid substance could be found in the joint cartilage in these two reports, where the diagnoses were primary amyloidosis and multiple myeloma

In our study, no known predisposition to amyloidosis was present in any of the cases and therefore we prefer to use the term CS GD for the compound found in the joint cartilage, at least until the results of histochemical and electron microsco pic studies of the compound are available

The tabulated relation to age indicates that CS-GD accumulates in articular cartilage with age However the same characteristic age relation has been described for amyloid disease in many earlier reports (*Thung* 1957), (*Buerger and Braunstein* 1960) (*Wright et al* 1969), (*Pirani* 1976) The age relation is as mentioned statistically significant $(0.001 < P < 0.01)$

The fact that there is no difference between the content of CS GD in cartilage from the upper and lower parts of the articular surface of the femoral head presumably indicated that the substance is equally distributed all over the cartilage surface and that stresses such as pressure and friction cannot be if at all, the only factors in its pathogenesis Further the diffuse spreading inside the joint and the typical deposition just below the articular surface indicate that the substance is conveyed by the synovial fluid

The observation that amyloid material in the capsule was never found when the joint cartilage was negative for CS GD, may also be of some importance with regard to the relationship between the two substances

Electron microscopic investigations as well as chemical analysis designed to clarify this problem are in progress in our laboratory

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Acta path microbiol scand Sect A 88 61–68 1980

DIFFERENTIAL HISTOCHEMICAL STAINING OF GLYCOSAMINOGLYCANS IN THE MATRIX OF OSTEOARTHRITIC CARTILAGE

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Department of Orthopaedic Surgery Calcified Tissue Research Laboratory Rigshospitalet Copenhagen Denmark

Christensen S B & Reimann I Differential histochemical staining of glycosaminoglycans in the matrix of osteoarthritic cartilage Acta path microbiol scand Sect A 88 61–68 1980

The distribution of chondroitin sulphate and keratan sulphate in osteoarthritic cartilage is analysed in a histochemical study using Safranin O Alcian Blue-CEC and Toluidin Blue O at different pH values The material consisted of six femoral heads obtained during replacement surgery from patients with osteoarthritis and six control femoral heads from patients who were operated on because of either fracture or tumour The heterogeneous distribution of
from the osteoarthritic and control patients is described
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territorial staining of chondroitin sulphate especially around the cell clusters This was a constant finding even in cartilage with only slight osteoarthritic changes In the control patients this phenomenon was found only in the weightbearing area in very old cartilage or in cartilage with morphological signs of osteoarthritis Finally the cartilage of osteophytes contained practically only chondroitin sulphate These histochemical findings are in accordance with the increase in the rate of $^{35}SO_4$ incorporation despite the loss of glycosaminoglycans in osteoarthritis as described by others and they reflect probably an accelerated synthesis of glycosaminoglycans

Key words Articular cartilage osteoarthritis glycosaminoglycans

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Hyaline articular cartilage is a highly specialized connective tissue characterized by the matrix content of collagen and proteoglycans These proteoglycans are composed of a central core to which a large number of negatively charged polydimeric sugar chains are attached giving macromolecules stiffly extended in space with an enormous negative field (*Rosenberg et al* 1970 *McDevitt* 1973) Aggregates of proteoglycans linked together by hyaluronate and stabilized by a glucoprotein component (*Hardingham & Muir* 1972 *Hascall & Heinegård* 1974 *Heinegård & Hascall* 1974) are responsible for the resiliency and resistence to deformation of the cartilage This ability of the cartilage to act as a shock absorber becomes weakened when glycosaminoglycans are depleted resulting in an impaired biomechanic function of the joint (*Kempson et al* 1970 *Harris et al* 1972)

In osteoarthritis biochemical studies have detected a decrease in glycosaminoglycan levels on dry weigth hexosamine concentration (*Matthews* 1953 *Mankin & Lippiello* 1970 1971 *Bjelle et al* 1972 *Lust & Pronsky* 1972) but parallel with the reduction an increased production of glycosami

TABLE 2

Carcinoma type	No of cases	Mean diameter cm	Per cent with axillary metastases
Tubular carcinoma ++++	39	0 8	10 8
Tubular carcinoma +++	56	1 4	31
Tubular carcinoma ++	50	1 7	36
Tubular carcinoma +	70	2 0	51
Tubular carcinoma 0	47	2 5	52

proportion of the tumours were small it is well known that pure tubular carcinoma is always a small lesion (*Taylor & Norris* (14) *Carstens* (2))

The low frequency in old materials can be explained if the tubular carcinomas progress to another type *Carstens* (2) has emphasized that the larger tubular carcinomas have a component of a different histologic type *We have found that more than half of the tubular* carcinomas have a more or less prominent zone of a more undifferentiated (»ductal«) type in their periphery We call the pure tubular carcinomas »tubular carcinoma ++++« and the latter type »tubular carcinoma +++« Further histologic studies disclosed a third type which was mainly »ductal« but contained a distinct small proportion of tubular structures (»tubular carcinoma ++«) and a fourth type with *only occasional tubules* mostly located in the central part of the lesion (»tubular carcinoma +«) The tumours described above have a *rather distinctive histologic structure with comparatively* small relatively inconspicuous cribriform or solid intraductal proliferates and a striking sclerotic central part with abundant elastoid tissue On these grounds we were able to discern still another type of »ductal« carcinoma which was devoid of tubules (»tubular carcinoma 0«) yet probably derived from tubular carcinoma

We thus believe – on histologic grounds – that the different types of tumours described here represent stages in a gradual progression to a less differentiated neoplasm (*i e* tubular carcinoma ++++ → *tubular carcinoma 0)* This is further supported by statistic evidence A grouping of the cases according to the afore mentioned principles is given in Table 2

These data strongly suggest that the histologic grouping really describes a progression to tumours with increasing size and higher frequency of nodal metastases

In our original group of ductal carcinomas N O S there remains 112 cases (22 5% of the whole material) which cannot be included in the aforementioned groups This remainder represents tumours which we designate as invasive »comedo« carcinomas They are characterized by rather large atypical cells widespread intraductal growth and out growth in the lobules (»cancerisation of lobules«) Elastoid stromal tissue is inconspicuous or absent in these tumours and they evidently progress from intraductal carcinomas This type of duct carcinoma is clearly defined by *Hultborn & Tornberg* (8)

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[illegible]

reduction was found to be proportional to the severity of the disease (*Mankin et al* 1971)

The histochemical reaction in these studies was based on the total amount of polyanions in the cartilage

The intention of the present study was to apply to osteoarthritic cartilage histochemical methods which permits differential staining of chondroitin and keratan sulphate sulphated and carboxylated glycosaminoglycans

MATERIALS AND METHODS

Cartilage specimens from 12 femoral heads were used In the osteoarthritic group six heads were obtained during replacement surgery (mean age 64 range 50–79 years) In the control group five heads were obtained from patients with fracture of the femoral neck and one from a patient with a tumour in the knee who was exarticulated at the hip joint This patient (age 22 years) was included in order to increase the range of age (mean age 69 range 22–88 years)

The specimens were fixed in 4% neutral buffered formaldehyde for 8 weeks and decalcified in 22% formic acid with 10% sodium citrate for 3 weeks (*Morse* 1945) Following decalcification the specimens were washed in running tap water dehydrated in graded alcohol cleared in methylsalicylate and double embedded in celloidin paraffin Sections were cut at 6 micra

The paraffin sections were deparaffinized in xylene hydrated through graded alcohols to distilled water stained hydrated again through alcohols cleared in xylene and mounted in Eukitt

Staining Methods

1 Safranin O fast green iron haematoxylen (*Lillie* 1965) In permanently mounted sections this dye is transformed to the orthochromatic form where it obeys Beer s law and can be used in quantification of polyanions (*Rosenberg* 1971) The Safranin O stained sections were used to classify the degree of osteoarthrotic changes in an area in the cartilage according to the histological histochemical grading system of *Mankin et al* (1971)

2 Alcian Blue with the critical electrolyte concentration (CEC) principle (*Scott and Dorling* 1965)

Sections were stained with Alcian Blue 8GX (I C I) 0 05 per cent in 0 025 M acetate buffer pH 5 8 containing 0 4 or 0 9 M $MgCl_2$ In control studies concentrations of 0 05 0 ? 0 5 0 6 0 8 1 0 1 2 and 1 5 M $MgCl_2$ were also used The sections were stained for 24 hours at room temperature In cartilage Alcian Blue 0 4 M $MgCl_2$ will stain both chondroitin 4/6 sulphate and keratan sulphate while at 0 9 M $MgCl_2$ only staining of high molecular weight keratan sulphate remains (*Laurent & Scott* 1964 *Scott* 1967)

3 Metachromatic staining with Toluidine Blue at different pH values Sections were stained for one minute in 0 01 per cent Toluidine Blue O (Merck) in Walpole buffer (pH 1 0 2 0 3 0 4 0 5 0) The interaction between cationic dyes and polyanions is of electrostatic nature and as the pK values of the carboxyl group and the sulphate group are different the reaction will depend on the hydrogenic ion concentration in the medium Metachromasia persisting at pH 1 5 indicates the presence of sulphated glycosaminoglycans while carboxyl groups will loose their metachromasia at pH 3 (*Krygier & Kasprzyk* 1961 *Spicer* 1962)

Chemical Modification Experiments

1 Active methylation in 0 1 N HCl in methanol for 4 hours at 56 °C (*Lillie* 1958) This procedure should abolish the metachromasia since methylation removes the sulphate groups with formation of free methyl sulphate esters and since carboxyl groups become esterified

2 Methylation saponification sequence Methylated sections were incubated in 1 per cent KOH in 70 per cent alcohol at 22 °C for 30 min (*Spicer* 1960) The methyl esters are removed and metachromasia is now based only on carboxyl groups

3 Oxidative deamination with ninhydrin (0 5 per cent in absolute alcohol) at 37 °C for 30 min (*Pearse* 1960 *Yosuma & Ichikawa* 1953) This was done to elucidate any competitive effect of protein especially at low pH values (*Andersen & Ehlers* 1967)

4 Enzyme digestion experiments Sections were digested with 0 1 per cent bovine testicular hyaluroni

sulphate but not keratan sulphate dermatan sulphate and heparitin sulphate (*Meyer et al* 1956 *Leppi & Stoward* 1965)

In order to control the effect of the fixation and decalcification procedures on the different staining methods specimens from non calcified costal cartilage obtained post mortem from a young person were stained

3

3

weeks decalcification

The intercellular matrix is divided into zones according to the relation to the cell lacuna In this paper the area surrounding one cell is named territorial and the interterritorial matrix is the area between territorial zones In gross terms the articular cartilage is composed of the tangential layer at the surface the intermediate the radiate and the calcified layer beneath the tidemark

RESULTS

Osteoarthritic Cartilage

The degree of cartilage degeneration evaluated on the basis of the Safranin O stained preparation varied considerably from site to site within the same femoral head Pronounced changes were seen in

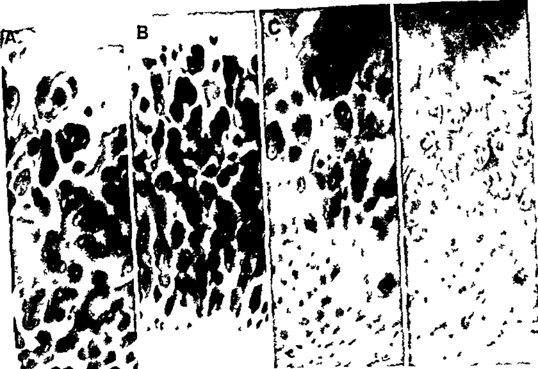


Figure 1 *A* Safranin O *B* Toluidine Blue pH 1 *C* Alcian Blue 0 4 M $MgCl_2$ *D* Alcian Blue 0 9 M $MgCl_2$ (serial sections) Magnification × 100 Osteoarthritic cartilage (grade 8) from the femoral head of a patient aged 50 years Superficial loss of staining intensity by Safranin O and an intense territorial staining is seen (A) By Toluidine Blue at pH 1 staining of sulphated glycosaminoglycans is present (B) and by Alcian Blue 0 4 M MgCl this staining of territorial glycosaminoglycans is seen especially high around cell clusters (Arrows) However with Alcian Blue 0 9 M $MgCl_2$ (D) the staining has disappeared indicating the territorial glycosaminoglycans to be chondroitin sulphate

weightbearing areas whereas less severe changes were present in non weightbearing areas The reduction in the staining ability was first seen and most distinctly at the surface (Fig 1A 4A)

The osteoarthritic cartilage was characterized not only by loss of staining for glycosaminoglycans but also by increasing territorial staining with Alcian Blue 0 4 M $MgCl_2$ especially around cell clusters (Fig 1C 4B) This was also seen in cartilage with slight osteoarthritic changes

By staining with Alcian Blue 0 9 M $MgCl_2$ mostly interterritorial staining was seen except for the most basal part of the radial zone and the calcified cartilage where territorial staining was present (Fig 1D) The area around the cell clusters showed insignificant staining with Alcian Blue 0 9 M MgCl but contained sulphated glycosaminoglycans as shown with Toluidine Blue at low pH values (Fig 1B)

Furthermore is was observed that the cartilage on the osteophytes was hardly stained at all with Alcian Blue 0 9 M MgCl (Fig 2)

Control Cartilage

The distribution of glycosaminoglycans in matrix varied in this group two different patterns were observed

One group (cartilage from 3 patients aged 22 69 and 73 years) showed the following features By Safranin-O staining the histological histochemical grading showed no significant signs of osteoarthritis and especially no loss of staining ability (Grade 0–2) (Fig 3A)

Staining with Alcian Blue 0 4 and 0 9 M $MgCl_2$ confirmed these findings the highest staining intensity was seen interterritorially at the surface and in the territorial zone in the basal part of the cartilage (*Fig 3CD*) However staining at 0 9 M $MgCl_2$ differed from staining at 0 4 M $MgCl_2$ At 0 9 M $MgCl_2$ it was seen that the area with the highest intensity was located more basally than by staining at 0 4 M $MgCl_2$ (Fig 3CD) Staining by Toluidine Blue showed at pH 1 the degree of sulphated glycosaminoglycans was increased basally

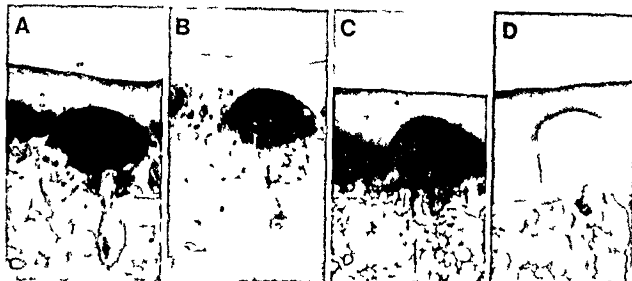
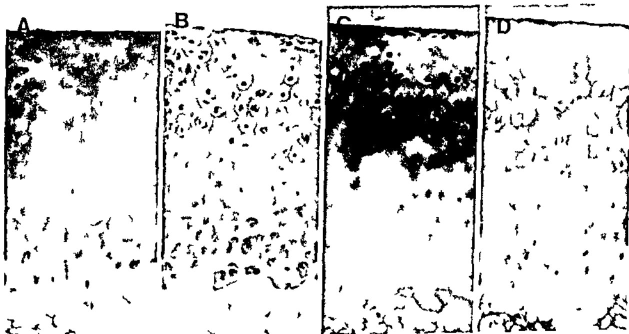


Figure 2 *A* Safranin O *B* Toluidine Blue pH 1 *C* Alcian Blue 0 4 M $MgCl_2$ *D* Alcian Blue 0 9 M $MgCl_2$ Magnification ×25 Osteophyte cartilage from a patient aged 68 years The glycosaminoglycans in the osteophytes cartilage are mainly chondroitin sulphate as metachromatic staining with Toluidine Blue at pH 1 (B) disappeared after *hyaluronidase digestion and staining with Alcian Blue showed very low intensity at 0 9 M $MgCl_2$ (D)*

In the other group of control cartilage (from 3 patients aged 72 87 and 88 years) the staining with Alcian Blue 0 9 M $MgCl_2$ did not differ from the group mentioned above but in the weightbearing area there was increased territorial staining with Alcian Blue 0 4 M $MgCl_2$ The cartilage from the *patient aged 72 showed a smaller area with* unmistakable degenerative changes (grade 5) in Safranin obtained preparation

Chemical Modification Experiments

Following active methylation the metachromatic reaction with Toluidine Blue at pH 5 disappeared *After saponification no colour reaction was seen*



... e pH 5 *C* Alcian Blue 0 4 M $MgCl_2$ *D* Alcian Blue 0 9 M $MgCl_2$ ... No ... f the ... te is

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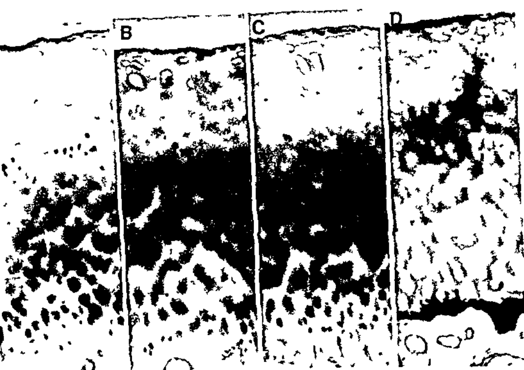


Figure 4 *A* Safranin O *B* Alcian Blue 0 4 M $MgCl_2$ C Hyaluronidase digestion and Alcian Blue 0 4 M $MgCl_2$ *D* Methylation and saponification Toluidine Blue pH 5 Magnification × 100 Osteoarthritic cartilage from a patient aged 68 (grade 4) By Safranin O a superficial loss of staining is seen (A) By Alcian Blue 0 4 M $MgCl_2$ an increased territorial staining is shown even in the superficial area unstained by Safranin O This territorial staining disappear – except basally after digestion with hyaluronidase prior to staining with Alcian Blue 0 4 M $MgCl_2$ (C) this indicates the territorial glycosaminoglycan to be chondroitin sulphate After methylation and saponification sequences only orthochromatic interterritorial staining is present (D) This staining was unaffected by hyaluronidase digestion and is probably due to collagen staining

with Toluidine Blue at pH values below 4 but at pH 5 only interterritorial orthochromatic staining was seen both in osteoarthritic and control cartilage

In osteoarthritic cartilage some superficial loss of staining was also seen after these procedures (Fig 4D) and this staining was not altered by preceding hyaluronidase digestion

Oxidative deamination with ninhydrin was followed by slightly increasing metachromasia but the distribution of the reaction was the same

It was confirmed by digestion with bovine testicular hyaluronidase and subsequent staining with Alcian Blue 0 4 M MgCl that the glycosaminoglycans localized territorially in osteoarthritic cartilage was mainly chondroitin sulphate since loss of staining was seen following enzyme digestion (Fig 4BC) After hyaluronidase digestion no metachromatic staining with Toluidine Blue pH 1 was found in the osteophytes indicating that the sulphate groups in osteophytes cartilage (Fig 2B) derived from chondroitin sulphate

The investigations to control the effect of fixation and decalcification showed that the frozen sections had the highest staining intensity especially with Alcian Blue The critical electrolyte concentration for chondroitin sulphate in the decalcified sections was found at the same level as in the formalin fixated sections (between 0 6 and 0 8 M MgCl) The distribution in matrix was not changed and no difference was seen in preparations fixed for 8 days or 3 months After staining with Alcian Blue the decalcified preparations showed only a *thin light* staining at all surfaces both normal surfaces and surfaces cut during the preparation This surface staining was unaffected by high salt concentrations (1 5 M $MgCl_2$) and active methylation

DISCUSSION

Histochemical methods with selective precipitation of the polyanions by cationic dyes such as Alcian

Blue are relatively specific to characterize and locate chondroitin and keratan sulphate in cartilage Thus Alcian Blue as used in this study will stain both keratan sulphate and chondroitin sulphate at 0 4 M $MgCl_2$ but only keratan sulphate at 0 9 M $MgCl_2$ Hyaluronic acid may only contribute to the metachromatic reaction at pH above 4, but as the relative content is only about one per cent (*Hardingham & Muir* 1973, *Hascall & Heinegard* 1974) the metachromasia of hyaluronic acid is poor and as fixation with formalin probably will extract some hyaluronic acid as shown in other tissue (*Hale* 1946) it was suggested that hyaluronic acid did not contribute to the metachromatic reaction

In the present study increased territorial staining of chondroitin sulphate was found especially around the cell clusters in osteoarthritic cartilage This is in accordance with a production of glycosaminoglycans resembling that which is seen in immature cartilage as described in biochemical studies on osteoarthritic cartilage (*Mankin & Lippiello* 1971 *Bayliss & Ali* 1978) and it is consistent with the increased frequency of mitosis of the chondrocytes in osteoarthritis (*Hulth et al* 1972 *Telhag* 1972)

In normal cartilage a distribution of chondroitin sulphate and keratan sulphate corresponding to the one found in this study has been described (*Stockwell & Scott* 1965) However by staining aging cartilage with Alcian Blue 0 4 M $MgCl_2$ they found the most intense staining territorially In the present study this was only seen in cartilage from very old persons (aged 87 and 88) in the weightbearing area and from a patient with incipient degenerative changes Normal cartilage from patients of an age corresponding to »osteoarthritic age« (50–75 years) seemed to have the same distribution of glycosaminoglycans as younger adults Probably the increased territorial content of chondroitin sulphate is a very early sign of degenerative changes but not specific for osteoarthritis and it may perhaps occur in other joint diseases

Stockwell (1970) reported that the staining of the tangential layer by Alcian Blue 0 9 M $MgCl_2$ increased with age and degenerative changes and attributed it to substances similar to keratan sulphate The tangential staining by Alcian Blue found in this study was suggested not to arise from polyanions as this staining was unaffected by methylation

The interpretation of the methylated and saponificated sections (Fig 4D) is not clear The metachromatic reaction after these procedures may be based on carboxyl groups and as only interterritorial orthochromatic staining was seen in all layers of the cartilage carboxyl groups of chondroitin sulphate were not thought to contribute to the reaction The reason for this is probably diminished charge density after desulphatation where the necessary intercharge distance is exceeded (*Sylven* 1958)

As further hyaluronidase digestion prior methylation and saponification did not change the orthochromatic reaction at pH 5 it is agreed with *Spicer & Lillie* (1959) that the reaction in cartilage was based on staining of collagen However it remarkable that some superficial loss of staining was seen in osteoarthritic cartilage (Fig 4D)

Biochemical studies on normal and osteoarthritic cartilage have first of all shown that hyaline cartilage undergoes striking changes with increasing age (*Kaplan & Meyer* 1959 *Mathews & Glagov* 1966) In neonatal cartilage chondroitin 4 and 6 sulphate are the only glycosaminoglycans present approximately in equal proportions During development stage the composition changes from a low degree of sulphatation towards a complete degree of sulphatation of chondroitin sulphate and from a high ratio of 6 sulphate to 4 sulphate periods to a ratio of 0 25 or less in the fourth decade Keratan sulphate is found in cartilage within a few years after birth and rises to a plateau value in the fourth decade remaining approximately constant thereafter

In osteoarthritis the monomers are found to be smaller primarily due to alterations in the protein core The chondroitin sulphate rich region is smaller and the monomers appear to lack the hyaluronic acid binding region These monomers have lost their ability to bind to hyaluronic acid and form aggregates This results in a looser network of proteoglycans (*Inerot et al* 1978)

The decrease in glycosaminoglycans in osteoarthritic cartilage has been described as a loss of mainly chondroitin sulphate (*Bollet et al* 1963 *Bollet & Nance* 1966) keratan sulphate (*Mankin & Lippiello* 1971 *Benmaman et al* 1969 *McDevitt et al* 1973) or both (*Bjelle et al* 1972 *Kuhn & Leppelmann* 1958 *Lust & Pronsky* 1972) This discrepancy might be explained by the problems connected with tissue sampling In cartilage with minor degenerative changes and only superficial depletion of glycosaminoglycans primarily chondroitin sulphate is lost while in cartilage with severe depletion clusters of chondrocytes with high territorial contents of chondroitin sulphate the chondroitin sulphate/keratan sulphate ratios will be high

Samples from cartilage with slight and severe osteoarthritic changes might therefore have different chondroitin sulphate/keratan sulphate ratios

It is still not known whether the changes in the proteoglycan contents of osteoarthritic cartilage are due to increased degradation of normal proteogly-

cans by hyaluronidase (*Bollet* 1967 *Bollet & Nance* 1966) Catepsin D (*Dingle et al* 1971 *Sapolsky et al* 1973 *Ali & Evans* 1973) or neutral proteases (*Sapolsky et al* 1974) or whether the synthesized proteoglycans are altered (*Bayliss & Ali* 1978)

From the present study it is suggested that the synthesis mostly is characterized by an attempt to repair with a production modus of more immature nature

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PATTERN OF INTIMAL FOLDS AROUND INTERCOSTAL ARTERY ORIFICES IN RABBIT AORTA

A Reflection of Pattern of Blood Flow?

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Svendsen E Pattern of intimal folds around intercostal artery orifices in rabbit aorta A reflection of pattern of blood flow? Acta path microbiol scand Sect A 88 69–74 1980

The pattern of intimal folds was studied around intercostal artery orifices in rabbit aorta by scanning electron microscopy and light microscopy The area just proximal and distal to the orifices revealed a smooth surface lacking folds reflected by a stretched internal elastic membrane which showed no undulation Lateral as well as further proximal and distal well preserved folds were observed It is reasonable to suppose that the pattern of intimal folds in this area reflects the special flow conditions prevailing in and at the intercostal artery orifices

Key words Intimal folds intercostal artery orifices rabbit aorta

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Earlier reports have shown that there is reason to believe that blood flow has an influence upon the pattern of the intimal folds created by the internal elastic lamina in human and rabbit arteries (2 14) *Flaherty et al* (4) showed that even orientation of the endothelial cells depends on the local flow situation *Crawford et al* (2) observed that the formative influence of blood flow upon the folds in arteries of man was most striking at branching sites and bends

Svendsen and Jørgensen (14) showed compression of folds and shallow pits in the distal part of the minor curvature of the aortic arch and lateral to the intercostal artery orifices In the area between two intercostal artery orifices on the same side however a regular pattern of folds was seen (14 15) They suggested that the pits could be imprints of eddies

The aim of the present study was to obtain further information about the possible formative influence of blood flow upon the folds of the internal elastic lamina. For that purpose a study of the pattern of folds around the intercostal artery orifices in rabbit aortas as seen by light microscopy and scanning electron microscopy seemed appropriate since the flow in association with such orifices is known from model studies (5 9)

MATERIAL AND METHODS

6 *New Zealand* white male rabbits weighing about 3 5 kg each were used The animals were raised on a low fat diet without addition (Kanin Pellets Felleskjøpet Trondheim) Sacrifice was made by a perfusion technique described earlier (15) The blood was washed out with buffered *Tyrode* solution with pH 7 4 and osmolality of 300 mOsm/kg followed by perfusion with 2% buffered glutaraldehyde pH 7 4 for 5 minutes at a pressure of 80 mm Hg at room temperature Then the aortas were carefully dissected free and postfixed by immersion in buffered glutaraldehyde 2% for 2 hours dehydrated in increasing concentrations of alcohol and dried by the [illegible]

[illegible] microscopy was performed with a *Hitachi* HHS-2R scanning electron microscope After the scan

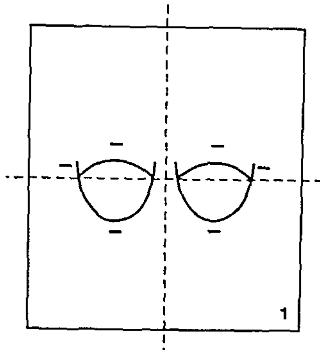


Fig 1 Schematic representation of a specimen from the area around intercostal artery orifices prepared for scanning electron microscopy The dotted lines indicate how the specimens were sectioned for light microscopy after removal from their stubs The bars indicate the sampled areas for light microscopy

ning electron microscopy had been accomplished the specimens were carefully removed from their stubs and samples for light microscopy were taken out with a sharp razor blade in a standardized manner from the sites shown in Fig 1 The samples were embedded in Epon and semithin sections were cut The sections were stained for elastin with *Victoria blue* and examined and photographed in a *Zeiss* photomicroscope

RESULTS

Grossly the same pattern of folds was seen in the second pair of the intercostal artery orifices (Fig 2) as in the eight pair (Fig 3)

Immediately proximally and distally to the intercostal artery ostia the intimal folds were lacking (Figs 2 and 3) The proximal area gave an impression of a smooth *infundibulum with lateral* folds which curved towards the ostium In proximal direction this area continued into regularly parallel folds (Figs 2 and 3) The extent of the smooth area downstream to the ostia could vary somewhat The smooth area merged with an area of regularly parallel folds still further distal (Figs 2 and 4) In some cases the folds just distal to the smooth area were somewhat wavy (Fig 3)

Light microscopy showed that the intimal folds were *mainly* dependent on folds in the internal elastic lamina In the smooth areas proximal and distal to the ostia the internal elastic lamina revealed almost no undulation (Figs 5 and 6) Lateral to the ostia, however, where marked folds appeared on scanning electron micrographs (Fig 4) a distinct undulation of the innermost elastic membrane was observed (Figs 5 and 6)

DISCUSSION

The present study has shown that the intimal folds are mainly created by the folds of the internal elastic lamina This is in agreement with previous work (14) It appears that the folds proximal and lateral to the ostia merge towards the ostium, following a stream-line pattern which should be expected in this area (9) The central part appears smooth This may reflect a disturbed flow situation possibly vortices which level the folds Flow experiments with model tubes have indicated this type of flow disturbance at such sites (7, 12)

Just distal to the ostia is an area where the special flow condition suggested at this site creates a small angle between the vessel wall and the tangent of the velocity profile This means increased dragging forces parallel with the vascular interface expressed as shear stress in the work of *Fry* (6) At this site folds seem to be lacking Further distally and proximally to the ostia the flow is expected to be regular and laminar (9) which may by reflected by regular parallel folds as shown in this paper and previous work (14) *Crawford et al* (2) also suggested formative influence of blood flow upon intimal folds demonstrated by casts of arteries in human bodies

There has been conflicting evidence as to whether intimal folds exist (1 2 10 13) or not (3, 8) *in vivo* This work does not give any definite answer to that question However, the pattern of the intimal folds suggesting an influence of blood flow upon the height and direction of the folds may be an indirect indication that intimal folds do exist *in vivo* Results obtained by *Svendsen & Tindall* (13) with arterial casts during continuous monitoring of the diameter

Fig 2 Scanning electron micrograph of one ostium from the second pair of intercostal arteries There is a longer distance between the ostia at this level than for the 8 pair Lateral to and between the ostia wellpreserved folds are seen No folds are present just proximal and distal to the ostium The arrow indicates the direction of blood flow 180×



Fig 3 Scanning electron micrograph of ostium from the 8th pair of intercostal arteries The very proximal and distal area to the ostium reveal a smooth surface Further proximally and distally as well as laterally wellpreserved folds are seen the distal ones somewhat wavy *The arrow indicates the direction of blood flow 110×*

of the abdominal aorta of rabbits under anesthesia however, strongly indicate their existence at least in the diastolic phase

Endothelial cell injury is commonly found at the lower lip of intercostal artery ostia (15 16) This area merges with the smooth area downstream to the ostia In the same area early development of atherosclerosis is seen (17) Mechanical stress may probably be one factor in introducing atherosclerosis (11)

In conclusion this work makes it reasonable to assume that the pattern of the intimal folds around intercostal artery orifices reflects the special flow conditions prevailing in and at the intercostal artery orifices

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Fig 4 Scanning electron m rograph of the 8th pair of intercostal artery orifices Note the smooth area proximal and distal to the ostia Note al that the crest between the ostia has a smooth appearance *Horizontal bars represent* regions sectioned for light microscopy Arrow indicates direction of blood flow 110×

Fig 5 Light micrographs of semithin sections from the indicated sites around the left hand ostium in Fig 4 P L and D indicate the proximal lateral and distal areas respectively Note the distended internal elastic membranes just proximal and distal to the ostium Laterally a marked undulation of the internal elastic lamina is seen Victoria blue stain 460×

Fig 6 Light micrographs of semithin sections from the indicated sites around the right hand ostium in Fig 4 P L and D indicate the same sites as mentioned in Fig 5 The same appearance of the internal elastic membrane as described in Fig 5 is see ctoria b 460×

A

P P'

L L'

D D'

6

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DIAGNOSTIC VALUE OF MODIFIED FINE-NEEDLE ASPIRATION BIOPSY OF THE KIDNEY IN GLOMERULAR DISEASES

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Helin H Diagnostic value of modified fine needle aspiration biopsy of the kidney in glomerular diseases Acta path microbiol scand Sect A 88 75–81 1980

43 renal fine needles aspirates obtained with a modified sampling technique were subjected to ultrastructural examination with the aim of assessing the diagnostic value of this method in various glomerular diseases Findings in the aspiration specimens consisting of solitary glomeruli were regarded as diagnostic in 35% of the cases When considered together with clinical data the fine needle aspiration biops es were suggest ve of diagnosis or provided valuable information in a further 49% of the cases Due to the small outer diameter of the fine needle 0 7 mm complications following this kind of biopsy are practically negligible Thus it offers a safe alternative to the conventional renal biopsy methods utilizing large bored cutting needles in cases where biopsies with these methods are contraindicated

Key words Fine needle aspiration renal biopsy glomerular diseases

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A modified fine needle aspiration method for biopsy of the kidney has recently been introduced for use in the diagnostics of glomerular diseases (8) With this technique single glomeruli can be obtained for electron microscopical examination The advantage of the fine needle method is its atraumatic nature for due to the small outer diameter of the biopsy needle (0 7 mm) it can be used without fear of clinically relevant complica tions in situations where conventional biopsy with one of the large bore needles is contraindicated

To assess the value of the morphological information obtained by this method 43 fine needle aspiration biopsies (FNA) from 42 patients were subjected to ultrastructural examination The resul ting information was correlated to that provided by data from clinical and laboratory investigations and in 29 out of the 43 cases to findings in parallel conventional biopsies

MATERIAL AND METHODS

43 consecutive FNA specimens from 42 patients with various clinical signs of renal disease (Table 1) were available for morphological evaluation The ages of the patients ranged from 5 to 74 years ($\bar{x}$ 34) Nine patients were under 15 years of age In 29 cases a control specimen from a conventional renal biopsy was exami ned

The FNAs were performed according to a previously described techn que (8) using a modified fine needle of 0 7 mm outer diameter The aspirates were processed according to a technically simple rapid sched le (1) [illegible]

[illegible] of light microscopy and stained with toluidine blue and Jones periodic acid silver methenamine Ultrathin sections were stained with lead citrate and uranyl acetate and examined in the JEM 100C electron microscope The FNA specimens were examined independently without knowing the results of the conventional biopsies performed simultaneously

The conventional biopsies were carried out with the Tru Cut® needle (Travenol Laboratories Inc Deerfield Illinois USA) One portion of the biopsy specimen was *embedded in paraffin and sectioned for light microscopy* and another processed for immunofluorescence examination

RESULTS

Indications of the biopsies are shown in Table 1 The *diagnostic information on the FNA specimens* was divided into four categories (Table 2) each of which is considered in greater detail in the following

TABLE 1 *Indications of 43 Renal Fine Needle Aspiration Biopsies*

nephrotic syndrome	3
acute renal failure	4
chronic renal failure	5
proteinuria	15
hematuria	8
diabetes with proteinuria	3
transplantation with renal failure	2
systemic lupus erythematosus	3
	Total 43

FNA establishing the diagnosis (15 cases) In this group the FNA as such was considered conclusive as to diagnosis These diagnoses were verified by light and immunofluorescence microscopical examination of the conventional biopsy specimens available in 9 cases Moreover a more precise morphological analysis was in some cases made possible by ultrastructural examination of the FNA specimens In the cases of membranous glomerulonephritis for example the stage of evolution of the membranous process could be accurately assessed (Fig 1) In 2 out of the 6 patients subjected to FNA only *membranous glomerulonephritis* was diagnosed Both patients presented clinically with SLE and marked proteinuria The FNA of a diabetic biopsied for proteinuria and renal failure showed the characteristic alteration of nodular diabetic glomerulosclerosis In a patient with rheumatoid arthritis heavy proteinuria and moderate renal failure the FNA revealed glomerular amyloidosis (Fig 2) FNA was performed on a renal transplant with moderately declined function in two patients In one of them the FNA finding was regarded as diagnostic of chronic rejection (Fig 3) In the other patient with Goodpasture s syndrome before transplantation *alterations consistent with this diagnosis* were seen in the FNA specimen

TABLE 2 *Diagnostic Information Provided by 43 Renal Fine Needle Aspiration Biopsies (FNA) (gn = glomerulonephritis)*

		Diagnosis or essential finding			
	biopsies	FNA with Tru Cut needle biopsy		FNA only	
Diagnosis established by FNA	15	membranous gn	4	membranous gn in SLE	2
		mesangiocapillary gn	1	nodular diabetic glomerulosclerosis	1
		minimal change nephropathy	1	chronic rejection	1
		acute › poststreptococcal ‹ gn	2	gn in Goodpasture s syndrome	1
		nodular diabetic glomerulosclerosis	1	*glomerular amyloidosis*	1
Diagnosis suggested by FNA	14	IgA nephropathy	6	residual changes after gn	1
		focal and segmental gn in SLE	1	focal glomerular sclerosis	1
		diffuse diabetic glomerulosclerosis	1	mesangioproliferative gn	1
		focal and segmental gn	1		
		residual changes after › poststreptococcal ‹ gn	1		
		focal glomerular sclerosis	1		
FNA providing useful information	7	normal ultrastructure	4	minor segmental changes	2
		minor segmental changes	1		
FNA providing little or no information	7	normal ultrastructure	3	normal ultrastructure	3
		unspecific scarring	1		
Total	43		29		14



Fig 1 a Portion of glomerular capillary wall in membranous glomerulonephritis in stage II This field of view shows subepithelial electron-dense deposits (D) with spikes (arrow) U = urinary space CL = capillary lumen × 22 400

1 b Section from a repeat fine needle aspiration biopsy of the same patient after 21 months A stage IV alteration is shown with deposition of electron lucent (D) and occasionally electron-dense (arrows) material within the widened basement membrane U = urinary space CL = capillary lumen × 16 800

FNA suggestive of a diagnosis (14 cases) Examination of the FNA specimens in these 14 patients provided information which when considered together with the clinical data was suggestive of a histopathological diagnosis Thus a mesangial sclerosing and proliferative glomerulonephropathy with characteristic electron-dense deposits suggested the presence of IgA nephropathy in 6 patients with either microscopic hematuria or recurrent macroscopic hematuria following infections (Fig 4) In the 11 patients with a parallel conventional biopsy a good correlation between the findings in these and the FNA specimens was observed Again the electron microscopical study of the FNA specimens allowed a more accurate analysis in some instances

Three patients were biopsied with FNA only One of them had a single kidney with moderate failure In the FNA specimen residual changes after glomerulonephritis suggested a previous acute glomerulonephritis agreeing well with the history

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		focal and segmental gn in SLE	1	focal glomerular sclerosis	1
		diffuse diabetic glomerulosclerosis	1	mesangioproliferative gn	1
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		unspecific scarring	1		
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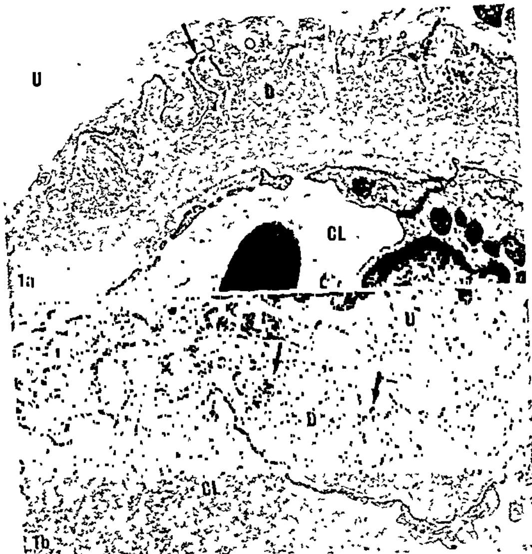


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Fig 2 Renal fine-needle aspiration biopsy from a patient with rheumatoid arthritis and nephrotic syndrome Note the subepithelial deposition of amyloid with spicule formation (arrows) in the glomerular basement membrane U = urinary space CL = capillary lumen × 14 600

Fig 3 This section shows a glomerular capillary loop in a 2 year-old renal allograft showing chronic transplant nephropathy Marked widening of lamina rara interna (arrows) of the basement membrane containing lamellae of newly formed lamina densa and endothelial cytoplasmic projections Capillary lumen (CL) considerably narrowed En = endothelial cells × 11 600



Fig 4 Section from a fine needle aspiration biopsy suggesting IgA nephropathy in a patient with recurrent macroscopic hematuria The field illustrates a glomerular mesangial area with large electron-dense deposits within the mesangial matrix (D) and beneath the glomerular basement membrane (arrows) × 6930

of the patient The remaining two patients both children 12 years of age were biopsied for proteinuria In one of them with a chronic course of corticoid resistant heavy proteinuria the FNA findings were suggestive of focal glomerular sclerosis and in the other with relatively mild proteinuria changes fitting well with the diagnosis mesangioproliferative glomerulonephritis were observed

FNA providing useful information (7 cases) This category consisted of cases in which the FNA finding offered an explanation for the clinical setting without having features specific enough to suggest a diagnosis Four of the 5 patients in this group biopsied with both methods had acute renal failure clinically In all FNA specimens the glomerular morphology was essentially normal Two of these four were suspected of epidemic nephropathy and in the other two the clinical data spoke in favour of tubulo interstitial rather than glomerular disease The conventional biopsies showed changes of acute tubulointerstitial nephritis in all 4 patients In the fifth case the FNA of a patient with rheumatoid arthritis and mild proteinuria revealed minor segmental changes in the mesangium including matrix increase and small deposits Focal and segmental glomerulonephritis was diagnosed in the control Tru-Cut biopsy

In two cases FNA was the only biopsy available Both demonstrated segmental mesangial alterations comparable to those described above The indication of the biopsy in both cases was mild asymptomatic proteinuria

FNA providing little or no information (7 cases) Included in this group were biopsies with normal morphology from 3 patients two of whom presented clinically with slight asymptomatic proteinuria and one with asymptomatic microscopic hematuria Out of these 3 patients only one a girl of 14 years with proteinuria showed mild segmental changes in less than 10% of the glomeruli in the conventional biopsy The glomeruli in the FNA of a fourth patient showed unspecific scarring Unclassifiable chronic glomerulonephritis was diagnosed in the parallel biopsy in this patient with chronic renal failure

In three children of 9 10 and 15 years with asymptomatic proteinuria less than 1 0 g/24 h the FNA performed as the only biopsy disclosed a normal glomerular ultrastructure This though expected (3) did not add to the information gained by clinical examinations

DISCUSSION

Percutaneous renal biopsy, though a relatively safe procedure (2, 13), is not without risks and should therefore be performed only when medical contraindications are not present. Of equal importance are ethical limitations, whereby biopsies for such as pure »scientific« purposes are not acceptable (11). To overcome the disadvantage of lacking morphological information in the cases mentioned, the present method has been developed for adoption in the study of glomerular diseases.

According to previous experiences (7, 8) the modified FNA method provides an adequate sample, consisting of 1 to 12 glomeruli, usually 1 to 4, in 79.1 % of cases. It is preferably used in diffuse glomerular diseases, which are known to affect all or nearly all glomeruli simultaneously (5).

As indicated by this study the information gained by the examination of FNA specimens was diagnostic in 35 % of the cases. These included diseases which present a characteristic ultrastructural picture. A further 49 % proved of value when the morphological information was correlated with that of clinical data. Correlation like this is also a known prerequisite for the successful use of fine-needle aspiration biopsy in diagnostic cytology (14). Normal findings in FNA specimens must be interpreted with care, because of the possibility of obtaining unaffected glomeruli in focal conditions. When electron microscopy is used however, some diseases, appearing focal in light microscopy, in fact show diffuse alterations. Thus for instance, the lesions in IgA nephropathy, a disease accounting for more than 25 % of all cases of primary glomerulopathies in several materials published (6) show diffuse distribution in electron microscopy (1). Diffuse ultrastructural changes have also been reported in focal glomerular sclerosis (10). As to the electron microscopical analysis in general it has been found to increase considerably the precision and objectivity of renal biopsy information (12).

The risk of complications involved in percutaneous renal biopsy has been shown to relate to the caliber of the needle used (15, 16). In this respect the modified fine needle with an outer diameter of 0.7 mm and with a sharp inner edge of 0.43 mm compares favourably with the commonly used large bore needles of 1.4–1.9 mm diameter. In 87 patients so far biopsied with the modified fine-needle only, no complications except transient microhematuria have been seen. This agrees well with Soderstroms's (14) experiences concerning renal aspiration biopsy with a fine needle (0.7 mm outer diameter) in cytodiagnostics. These biopsies like the 182 described by Pasternack et al. (9) and performed with a fine needle of the same caliber were not followed by any noteworthy complications.

The present FNA method is not meant to concur with the techniques generally used utilizing large bore needles. It is however a safe alternative when the last-mentioned can not be performed without exposing the patient to an undesired risk. An area of application of both clinical and scientific interest is repeat follow-up biopsy to assess the histological evolution of a disease process. Conditions without or with only minor renal symptoms following glomerular injury are seldom clarified by renal biopsy but are of undoubted interest. Such conditions are for instance malignant tumors or infectious diseases with secondary asymptomatic renal involvement.

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TRABECULAR BONE MINERALIZATION LAG TIME DETERMINED BY TETRACYCLINE DOUBLE-LABELING IN NORMAL AND CERTAIN PATHOLOGICAL CONDITIONS

FLEMMING MELSEN and LEIF MOSEKILDE

University Institute of Pathology and Medical Department III Aarhus Amtssygehus and Department of Neurology Aarhus Kommunehospital Aarhus Denmark

Melsen F & Mosekilde L Trabecular bone mineralization lag time determined by tetracycline double labeling in normal and certain pathological conditions Acta path microbiol scand Sect A 88 83-88 1980

Quantitative histomorphometric analyses of iliac crest biopsy specimens were performed after tetracycline double labeling in 41 normal individuals 20 hyper and 10 hypothyroid patients 18 patients with primary hyperparathyroidism 20 epileptic patients receiving long term anticonvulsant therapy and 17 patients after jejunoileal bypass for morbid obesity The mineralization lag time in trabecular bone or the period of time between apposition and subsequent mineralization of osteoid was calculated from the bone formation rate at BMU level (Basic Multicellular Unit) and the mean width of osteoid seams The mineralization lag time was 8–52 days (median 21 days) in normal individuals and showed no variation with sex or age The mineralization lag time was shortened in hyperthyroidism normal in anticonvulsant bone disease and in primary hyperparathyroidism and markedly prolonged in hypothyroidism and following jejunoileal bypass Among all individuals an inverse hyperbolic relation (r 0 94 $p<0 001$) was found between the mineralization lag time and the average cellular activity of the osteoblasts

Key words Mineralization lag time bone histomorphometry tetracycline double labeling

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Accepted as submitted 1 x 79

Unmineralized bone matrix is laid down by osteoblasts as osteoid seams on the bone surfaces That the osteoid seams have a certain thickness shows that there is a delay between the formation of bone matrix by osteoblasts and the subsequent mineralization During this period a sequential change in the staining properties of the osteoid is observed A distinct band which lies adjecent to the calcified matrix and stains with acid dyes has been termed the calcification front (*Matrajt et al* 1967) and has been taken as evidence of early mineralization Tetracycline antibiotics will deposit *in vivo* in these sites of active mineralization (*Milch et al* 1958 *Frost* 1969)

In a steady state situation with regard to osteoid thickness the rate of apposition of osteoid equals the rate of mineralization because any systematical change in this proport on [illegible]

[illegible]

T [illegible]

m[illegible]alization lag time or the time interval between apposition and subsequent mineralization of osteoid

The aim of the present investigation was to describe this calculation for trabecular bone and to report normal values Furthermore the mineralization lag time has been calculated in cert[illegible]

[illegible] for obesity

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Melsen F & Mosekilde L. Trabecular bone mineralization lag time determined by tetracycline double labeling in normal and certain pathological conditions Acta path microbiol scand Sect. A 88 83-88 1980

Quantitative histomorphometric analyses of iliac crest biopsy specimens were performed after tetracycline double labeling in 41 normal individuals 20 hyper and 10 hypothyroid patients 18 patients with primary hyperparathyroidism 20 epileptic patients receiving long term anticonvulsant therapy and 17 patients after jejunoileal bypass for morbid obesity The mineralization lag time in trabecular bone [illegible]

[illegible] no variation with sex or age The mineralization lag time was shortened in hyperthyroidism normal in anticonvulsant bone disease and in primary hyperparathyroidism and markedly prolonged in hypothyroidism and following jejunoileal bypass Among all individuals an inverse hyperbolic relation (r 0 94 p<0 001) was found between the mineralization lag time and the average cellular activity of the osteoblasts

Key words Mineralization lag time bone histomorphometry tetracycline double labeling

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Unmineralized bone matrix is laid down by osteoblasts as osteoid seams on the bone surfaces That the osteoid seams have a certain thickness shows that there is a delay between the formation of bone matrix by osteoblasts and the subsequent mineralization During this period a sequential change in the staining properties of the osteoid is observed A distinct band which lies adjecent to the calcified matrix and stains with acid dyes has been termed the calcification front (*Matrajt et al* 1967) and has been taken as evidence of early mineralization Tetracycline antibiotics will deposit *in vivo* in these sites of active mineralization (*Milch et al* 1958 *Frost* 1969)

In a steady state situation with regard to osteoid thickness the rate of apposition of osteoid equals the rate of mineralization because any systematical change in this proport[illegible] rance or [illegible] This assu[illegible] mineralization lag time or the time interval between apposition and subsequent mineralization of osteoid

The aim of the present investigation was to describe this calculation for trabecular bone and to report normal values Furthermore the mineralization lag time has been calculated in certain pathological conditions including hyper and hypothyroidism primary hyperparathyroidism anticonvulsant bone disease and ostemalacia following jejunoileal bypass for obesity

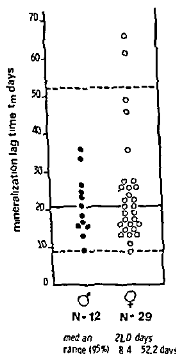


Fig. 1. Mineralization lag time (t_m days) in normal males and females. Median and normal range (95%) are indicated.

width of the osteoid seams ($^{a}W_f$) was normal because of an increase in the appositional rate of osteoid (= $^{s}V_{f(BMU)}$) and a simultaneous decrease in the mineralization lag time (t_m). The osteoblastic activity was decreased in the hypothyroid patients both at active cellular level and at BMU level. The mean width of the osteoid seams was reduced because of a marked decrease in the appositional rate of osteoid and in spite of a prolongation of the mineralization lag time.

The osteoblastic activity was slightly decreased in the hyperparathyroid patients at cellular level and at BMU level. The mean osteoid seam width was normal. The osteoid appositional rate was slightly reduced and the mineralization lag time was slightly but insignificantly, prolonged ($p < 0.10$).

The epileptic patients receiving anticonvulsants showed a normal osteoblastic activity at active cellualar level whereas the activity at BMU level was slightly increased. The mean osteoid seam width was increased because of the increased appositional rate of osteoid and in spite of the normal mineralisation lag time.

The osteoblastic activity was reduced in the intestinal bypass patients at both active cellular level and BMU level. The mean width of the osteoid seams was increased because of a marked prolongation of the mineralization lag time and in spite of a reduction in the appositional rate of osteoid.

TABLE 1. *Measured and Calculated Histomorphometric Values in Normal Individuals and in Certain Pathological Conditions*

		$S_{fract(f)}$ µm²/µm²	$S_{fract(lab)}$ µ²/µm²	$^{a}M/t$ µm/day	$^{s}V_{f(BMU)}$ µm³/µm²/day	$^{a}W_f$ µm	t_m days
Normal individuals	$\bar{x}$	0.179	0.135	0.65	0.50	9.6	23.5
	SE	0.011	0.010	0.02	0.02	0.4	2.5
Hyperthyroid patients	$\bar{x}$	0.173	0.144	0.94**)	0.86**)	9.3	13.1**)
	SE	0.019	0.013	0.05	0.08	0.6	1.1
Hypothyroid patients	$\bar{x}$	0.149	0.032**)	0.036**)	0.12**)	6.3**)	92.3**)
	SE	0.020	0.009	0.08	0.05	0.7	26.4
Hyperparathyroid patients	$\bar{x}$	0.281**)	0.204**)	0.56*)	0.41*)	9.9	28.6
	SE	0.023	0.019	0.03	0.03	0.6	2.8
Epileptics	$\bar{x}$	0.281**)	0.247**)	0.66	0.58*)	12.2**)	21.3
	SE	0.014	0.012	0.02	0.02	0.4	1.0
Intestinal bypass patients	$\bar{x}$	0.234*)	0.117	0.46**)	0.25**)	12.3**)	42.7**)
	SE	0.032	0.014	0.03	0.03	1.4	6.4

Significance of difference from normal mean: *) $p < 0.05$, **) $p < 0.01$ (Wilcoxon test for two pairs.)

MATERIAL AND METHODS

The investigation comprised the following classes of normal individuals and patients who had all given informed consent to participate

41 normal volunteers 12 males aged 23 to 56 years (mean 32) and 29 females aged 19 to 53 years (mean 29) Entry into the investigation was based on normal thyroid parathyroid kidney and liver function None received tranquilizers anticonvulsants or other chronic medication

20 patients with untreated hyperthyroidism aged 24 to 46 years (mean 33) and 10 patients with untreated primary hypothyroidism aged 33 to 75 years (mean 54) The diagnosis was based on clinical symptoms and signs measurements of total serum thyroxine serum tri iodo thyronine uptake test serum TSH and ^{131}I iodine uptake

18 patients with untreated primary hyperparathyroidism aged 29 to 74 years (mean 46) The diagnosis was based upon determination of serum immunoreactive parathyroid hormone (*Christensen* 1976) and total calcium corrected for individual variations in serum albumin In all cases a parathyroid adenoma was later found by neck exploration

20 adult epileptic outpatients aged 20 to 52 years (mean 29) who all received diphenylhydantoin some of them in combination with other anticonvulsants All had been treated for more than 10 years The patients were *selected from a group of 60 epileptics in whom bone* biopsy was performed (*Mosekilde and Melsen* 1976) The selection was based on the occurrence of an increased amount of osteoid and/or an increase in the mean size of the periosteocytic lacunes None of the patients had symptoms of bone disease

17 patients aged 29 to 64 years (mean 38) who 3 to 8 years (mean 5 2) previously had undergone jejunoileal bypass for morbid obesity The mean preoperative *weight was 121 kg (range 102–173 kg) During opera*tion 35 cm of jejunum was anastomosed end to side to 15 cm of the distal ileum The average weight loss was 39 kg (range 20–77) None of the patients had bone pains or *bone tenderness*

Transcortial iliac crest biopsies with a diameter of 8 mm (*Bordier et al* 1964) were obtained after doublelabeling with tetracycline (600 mg of demethyl chlortetracycline given orally 14 13 4 and 3 days before biopsy) The bone specimens were embedded undecalcified in methylmethacrylate 8 μm thick sections were cut and stained with Masson trichrome for quantification of osteoid (*Melsen et al* 1978) and 20 μm thick unstained sections were mounted for quantification of dynamic parameters by fluorescent microscopy (*Melsen and Mosekilde* 1978) The following measurements were performed in trabecular bone using the point count principle and simple measurements with a micrometric eyepiece*)

Fractional formation surfaces ($S_{fract(f)}$) μm²/μm² – the extent of osteoid covered surfaces in decimal fraction of the total trabecular bone surface

Fractional labeled surfaces ($S_{fract(lab)}$) μm²/μm² – the extent of single and double tetracycline labeled surfaces in decimal fraction of the total trabecular bone surface

Appositional rate ($^uM/t$) μm/day – the mean distance between the lines in all zones double labeled with tetracycline (^uM) divided by the interval of days (t) between the labelings This value expresses the bone formation rate at active cellular level uncorrected (u) for fortuitous obliquity of the plane of section**)

Mean width of osteoid seams (^uW_f) μm – the mean of four extreme almost equally spaced measurements in all surfaces covered with osteoid uncorrected (u) for obliquity of the plane of section

The following parameters were calculated

Bone formation rate BMU level surface referent ($^sV_{f(BMU)}$) μm³/μm²/day as

$$^sV_{f(BMU)} = \frac{S_{fract(lab)} \times {}^uM/t}{S_{fract(f)}}$$

which expresses the amount of new bone mineralized in unit time per unit osteoid covered surfaces In a steady state situation with regard to osteoid thickness $^sV_{f(BMU)}$ is numerical identical to the *appositional rate of osteoid* ($^sO_{f(BMU)}$ μm/day) defined as the average thickness of osteoid laid down in unit time and reflecting the average activity of active and inactive osteoblasts

Mineralization lag time (t_m) days (*Baylink et al* 1970) as

$$t_m = \frac{^uW_f}{^sV_{f(BMU)}}$$

This calculation is based on the relations

$$^uW_f = t_m \times {}^sO_{f(BMU)},\quad {}^sO_{f(BMU)} = {}^sV_{f(BMU)}$$

which express that the mean width of osteoid seams at steady state equals the mean thickness of osteoid laid down per day multiplied by the average number of days with osteoid formation before mineralization starts

RESULTS

The individual values of the mineralization lag (t_m) from the normal males and females are shown in Fig 1 No significant differences were found between the sexes ($p > 0.10$ (Wilcoxon test)) and t_m was unrelated to age (R = 0 28 $p > 0.10$ (Spearman

Table 1 gives the arithmetric mean values (± SE) of t_m and of the parameters involved in the calculation of t_m in the normal individuals and in *the different patient groups* The distribution of the individual t_m values in the different groups is shown in Fig 2

The hyperthyroid patients showed an increased activity of the osteoblasts both at active cellular ($^uM/t$) and at BMU level ($^sV_{f(BMU)}$) The mean

Among all individuals a highly significant inverse hyperbolic relation ($r = 0.94$, $p < 0.001$) was found between the mineralization lag time and the activity of the osteoblasts at BMU level (Fig 3). A similar but less significant relation ($r = 0.75$, $p < 0.01$) was found between the mineralization lag time and the osteoblastic activity at active cellular level.

DISCUSSION

The present study has demonstrated that the mineralization lag time in trabecular bone in normal individuals is 8 to 52 days with a median of 21 days. This period of time is longer than the 10 days previously reported by *Lacroix* (1970) who analyzed active bone formation surfaces in cortical bone. It should however be noticed that the mineralization lag time in the present study represent an average value for both active (tetracycline labeled) and inactive (osteoid covered unlabeled) bone formation surfaces because both the bone formation rate and the mean width of osteoid seams are averaged over the total surface covered with osteoid. This calculation gives more information about the background of variations in the observed mean width of osteoid seams than determinations at active bone formation surface alone.

Both the appositional rate ($^{u}M/t$) and the mean width of osteoid seams ($^{u}W_{o}$) were uncorrected for fortuitous obliquity of the plane of section. This correction involves however a multiplication of both parameters by 0.73**) and will therefore not change the calculated mineralization lag time.

It appears from the present study that excess thyroid hormone(s) shortens and deficient thyroid hormone(s) prolongs the mineralization lag time. This is in accordance with the finding that thyroid hormone( )

however contribute to the shortened mineralization lag time by inducing mineralization at an earlier stage of the maturation of osteoid.

The bone changes in primary hyperparathyroidism are among other factors characterized by an increased amount of osteoid (*Melsen et al* 1977, *Meunier et al* 1978) because of an increased extent of osteoid covered surfaces. The increase in the amount of unmineralized bone is not due to a mineralization defect because of the normal mean osteoid seam width and the normal mineralization lag time. The increased extent of bone forming surfaces may be explained by the high bone turnover with an increased number of remodeling units and the longer life span of bone forming sites (*Melsen et al* 1977, *Meunier et al* 1978).

The observed normal mineralization lag time in anticonvulsant bone disease and the markedly prolonged lag time in intestinal bypass patients supports the concept that anticonvulsant bone disease differs from vitamin D deficient states (*Schultz and Delling* 1974, *Mosekilde et al* 1977). This is in accordance with the findings of low levels of 1,25-dihydroxycholecalciferol, the ultimate metabolically active vitamin D metabolite, in intestinal bypass patients (*Lund et al* 1979) and slightly increased values in epileptic patients receiving anticonvulsants (*Jubiz et al* 1977).

In the present study a high cellular activity of the osteoblasts was associated with a short mineralization lag time and vice versa. This supports the concept that the osteoblasts on the bone surface are in some way associated with the deposition of mineral at the calcification fronts (*Arnot and Pautard* 1967, *Bonucci* 1971).

The calculation of the mineralization lag time is based on a steady state situation with regard to bone remodeling. The bone remodeling period (sigma) or the period of time between the activation of new remodeling unit and its completion of the resorptive and subsequent formative phases equals the shortest time following a stimulus that a new steady state characteristic for that stimulus can exist (*Frost* 1969). The normal bone remodeling period in iliac crest trabecular bone can be estimated to about 4 months from the mean thickness of completed remodeling units (*Lips et al* 1978), the bone formation rate at BMU level (*Melsen and Rosenkilde* 1978) and the proportion of formative to resorptive surface (*Melsen et al* 1978). This period of time in trabecular bone equals the previously reported s...

osteoid seam and less rapid at the end (*Frost* 1962, *Lacroix* 1970). In order to avoid sampling errors a relatively large number of osteoid seams should therefore be examined in each individual or in each test group.

We conclude that the mineralization lag time is a

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*) The symbols and notations used in the paper are to a great extent identical with those suggested by *H. M. Frost* (1977).

BMU – Basic Multicellular Unit. The functional entity of cells responsible for the turn-over of a remodeling unit of bone (*Frost* 1969).

**) An orientation correction factor (0.73) has been computed by *H. M. Frost* (Cosine integration between variable bounds) on Hewlett Packard HP 65 program.

Mineralization lag time, t_m days

300
200
100
80
60
40
20
0

Normal individuals | Hyperthyroidism | Hypothyroidism | Primary hyperparathyroidism | Epileptics receiving anticonvulsants | Intestinal by pass

p < 0 01 | p < 0 01 | n s | n s | p < 0 01

Fig 2 Distribution of t_m values in normal individuals and in various pathological conditions The dotted lines indicate the arithmetic mean values

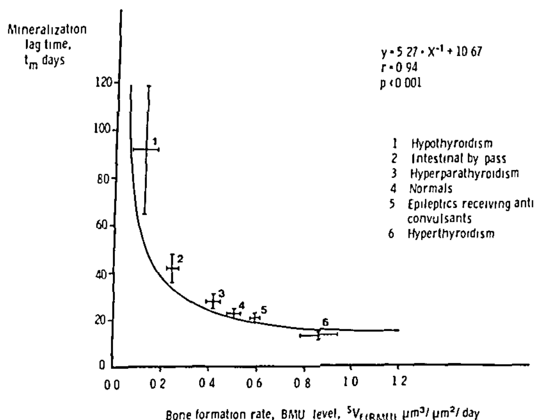


Fig 3 The relation between the mineralization lag time and the bone formation rate at BMU level in normal and pathological states Mean and standard error of the mean are indicated for the different groups investigated

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XANTHOGRANULOMATOUS PYELONEPHRITIS

A Clinico pathological Study with Special Reference to Pathogenesis

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Møller J C F & Kristensen I B Xanthogranulomatous pyelonephritis A clinico pathological study with special reference to pathogenesis Acta path microbiol scand Sect A 88 89-96 1980

Nineteen cases of xanthogranulomatous pyelonephritis (XP) were studied There was a close relation to pyonephrosis and urinary tract obstruction was present in all cases but one which represented the tumorous variety of XP The degree of xanthogranulomatous involvement was correlated to the severity of the acute inflammatory affection as evidenced by the clinical findings and the urographical excretory function The topography of the xanthogranulomas was related to the level of urinary tract obstruction in accordance with the pattern of reaction common to obstructive pyelonephritis Haemorrhage and considerable fibrous thickening and stenosis of arteries surrounding the xanthogranulomatous foci were conspicious features Based upon a morphological staging it was shown that the structural changes (towards fibrosis) were parallelled by a series of cellular events indicating removal of intracellular lipids in xanthoma cells by transformation to lipofuscin and transport in PAS-positive interstitial macrophages This was comparable to the findings in experimental XP suggesting a similar and simple way of pathogenesis It is concluded that XP – with the possible exception of the tumorous variety – can be ascribed to local resorption phenomena caused by concurrent factors usually involved in pyelonephritis urinary tract obstruction being the essential precondition focal haemorrhage and ischaemia contributary elements

Key words pyelonephritis xanthogranulomatous clinicopathological study pathogenesis

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Accepted as submitted 9 x 79

The term xanthogranulomatous pyelonephritis (XP) denotes a morphological sub-type of pyelonephritis which is characterized by the presence of yellowish nodules and pericalyceal streaks of granulation tissue the essential feature of which is lipid laden macrophages In spite of the unusual and often impressive appearance which may cause confusion with renal carcinoma (*Selzer et al* 1957) it appears from both casuistic reports and large retrospective series (*Gingell et al* 1973 *Gammill et al* 1975 *Malek & Elder* 1978) that the clinical findings and the pre-disposing factors are not fundamentally different from those of suppurative nephritis in general The mechanisms involved in the xanthogranulomatous reaction are not known in detail but prolonged suppuration in addition to urinary tract obstruction have been suggested as the most likely causal factors (*Selzer et al* 1957, *Friedenberg & Spjut* 1963)

The present work was undertaken in order to further investigate the basis for the xanthogranulomatous reaction by 1) correlating clinical and pathological findings and 2) comparing the morphological changes in the xanthogranulomas with studies in experimental models (*Povysil & Konickova* 1972)

MATERIALS AND METHODS

Nineteen cases representing one autopsy and eighteen nephrectomy specimens were studied Thirteen of these had been previously diagnosed and recorded at six institutes of pathology An additional six cases were

characteristic feature of different metabolic bone diseases and that observed changes in the mean width of osteoid seams may be explained by variations in the appositional rate of osteoid and in the mineralization lag time

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Fig. 1 Early xanthogranulomatous reaction (stage I). Large vacuolated and granulated xanthoma cells mixed with polymorphonuclear leucocytes

Fig. 2 Intermediate xanthogranulomatous reaction (stage III). Sheets of xanthoma cells among slender capillaries. Rather few inflammatory cells

collected prospectively representing 5 4 per cent of all nephrectomies performed during one year

The extent of xanthogranulomatous involvement was macroscopically graded according to the following criteria

I Slight degree of involvement	focal changes in the pericalyceal renal tissue
II Moderate degree of involvement	diffuse pericalyceal changes (i e limited to the renal medulla)
III *Severe degree of involvement*	*changes involving the whole kidney*

Paraffin sections were stained with haematoxylin eosin van Gieson PAS Perls Prussian blue and Ziehl Neelsen modified *for staining of lipofuscin*

For comparison with experimentally produced XP a morphological staging was done based upon the well *known pattern of inflammatory reactions in general and upon the presumed dynamics of human XP in particular* (*Tonelli & Gianotti* 1969 *Putschar* 1934)

Stage I	maximal necrosis and granulocytic infiltration Xanthoma cells loosely arranged (Fig 1)
Stage II	xanthoma cells organized in more solid formations along capillaries Sporadic necroses
Stage III	xanthoma cells closely packed in a well vascularized stroma (Fig 2)
Stage IV	decrease and splitting up of xanthoma cells due to fibrosis (Fig 3)

In each stage the staining reaction for PAS positive substances lipofuscin and iron was given a score from 0 to + + + 0 representing no or doubtful positive reaction + + + maximal reaction

RESULTS

Clinical observations Relevant data are recorded in Table 1 There was a slight female preponderance (11 ♀/8 ♂) and a mean age of 56 years at the time of nephrectomy

Eight patients had no previous symptoms of urinary tract disease and nephrectomy was performed after a relatively short period of acute illness with septic fever and flank pain In 9 patients nephrectomy was preceded by several years of urinary tract disease and signs of acute pyelonephritis were present in only half of the cases

Three patients had or developed malignancy of the urogenital tract (carcinoma of the cervix uteri (1) carcinoma of the bladder (1) and carcinoma of the ureter (1) One patient developed XP in relation to pregnancy and one patient was diabetic

TABLE 1 *Xanthogranulomatous Pyelonephritis Clinical Summary*

Case no	Age (years)	Sex	Chronic urological disease	Acute disease (duration)	Obstructive lesion	Bacteriological findings (urine)
I	73	M	+	6 weeks	Ureteral stone	Unknown
II	64	M	–	3 months	Ureteral stone	Gram – rods
III	55	F	+	–	Pelvic stone	Gram – rods
IV	72	M	+	–	Carcinoma of the bladder	Gram + rods
V	58	F	+	–	Pelvic stone	Proteus
VI	81	M	–	5 weeks	Pelvic stones	Proteus/E coli
VII	30	M	–	4 weeks	–	Gram + cocci
VIII	70	M	+	–	Pelvic stone	Gram – rods
IX	22	F	–	4 months	Pelvic stone	Unknown
X	79	F	–	2 weeks	Pelvic stone	Unknown
XI	69	F	?	?	Ureteral stone	Unknown
XII	61	F	–	4 weeks	Ureteral stone	E coli
XIII	40	M	–	4 months	Aberrant vessel	Unknown
XIV	8	F	+	4 weeks	Uretero pelvic stenosis	E coli
XV	67	F	+	3 months	Pelvic stone	E coli
XVI	65	F	+	3 months	Pelvic stone	E coli
XVII	52	F	?	?	Ureteral carcinoma	E coli
XVIII		F	+	–	Pelvic stones	E coli
XIX	26	M	–	4 weeks	Pelvic stone	Gram – rods



Fig 4



Fig 5



Fig 6



Fig 7

Fig 4 Part of kidney with typical pericalyceal xanthogranulomatous changes Note haemorrhage in relation to xanthogranulomatous foci

Fig 5 Focal «tumefactive» type of XP

Fig 6 Marginal xanthogranulomatous area with accumulation of macrophages giving the red brown PAS reaction of lipofuscin

Fig 7 Section of xanthogranuloma with areas of haemorrhage and conspicuous amounts of haemosiderin in xanthoma cells and other macrophages (Perls Prussian blue)

in staining intensity between central and marginal areas

The various staining reactions as correlated to stage are illustrated in Fig 8

Clinico pathological relations From Table 2 it appears that in 3 groups of patients differing as to clinical course in most cases the presence of the acute phase was correlated to a high degree of xanthogranulomatous involvement and vice versa No correlation was found between the clinical course (the duration from onset of clinical symptoms to nephrectomy in particular) and the microscopic stage of xanthogranulomatous change

Seventy five per cent of kidneys with moderate and severe degree of xanthogranulomatous involve

TABLE 2 *Quantitative Xanthogranulomatous Changes in Relation to Clinical Course of Urological Disease*

	Macroscopic degree of renal involvement		
	I	II	III
Acute disease only (< ½ year) (8)	0	3	5
Chronic disease with acute phase (4)	0	1	3
Chronic disease without acute phase (5)	3	1	1
Unknown clinical course (2)	1	0	1

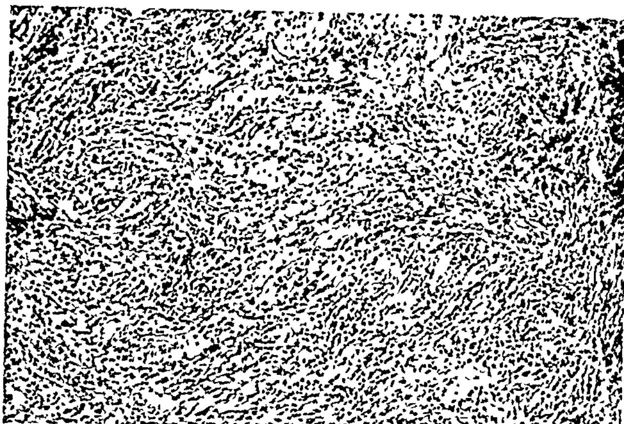


Fig 3 Advanced xanthogranulomatous reaction (stage IV) Scattered xanthoma cells in a fibrillary stroma

Bacteriuria was present in the majority of cases and E coli was the organism most frequently isolated Significant bacteriuria ($> 1 \times 10^5$ *colonies/ml)* however was present only in a minor part of the series Urinary tract obstruction was demonstrable in all cases but one This was usually due to pelvic and ureteral *stones (74 per cent)*

An excretory urogram (IVP) was obtained in only 8 cases since 11 kidneys showed no function

On account of the radiological findings renal tumour was suspected in 6 cases

Pathological findings Typically the xanthogranulomatous kidney was enlarged 17 cases presenting varying degrees of pyonephrosis The xanthogranulomatous changes varied from focal festoon like yellowish streaks or easily missed small nodules in the pericalyceal renal tissue to more generalized lesions Haemorrhage in relation to the xanthogranulomatous foci was a conspicious feature of several cases (Fig 4)

One case (no VII) represented the localized tumourous form of XP (Fig 5)

Microscopic examination of renal tissue adjoining the xanthogranulomatous foci showed signs of acute and *chronic interstitial* nephritis in all cases There was an obvious predilection of the xanthogranulomas [illegible] segments with the most severe *inflammation* [illegible] on – especially in relation to the pelvic fornices and renal papillae

Varying and at times pronounced degrees of luminal narrowing due to intimal thickening and fibrosis were observed in arteries situated at the periphery of the xanthogranulomas Venous changes or thromboses were not seen

The xanthogranulomas showed only slight mu*tual structural variation which made* a morphological staging of the individual case possible

Initially (stage I) the xanthoma cells appeared vacuolated and contained mostly small PAS positive granules and partly degraded cellular elements (mainly polymorphonuclear leucocytes) Later (stage III) the PAS positivity and the phagocytized elements disappeared, and the cytoplasm appeared more homogeneous (compare Fig 1 and 2)

Macrophages differing from typical *xanthoma* cells due to a slightly smaller size absence of cytoplasmic vacuoles and a more pronounced PAS positive reaction were observed *sporadically* at stage I and mainly in the central xanthogranulomatous area *These cells made up a more conspicious* feature of stage II At stage III macrophages of a similar type appeared in clusters at the periphery of the xanthogranulomas and showed the staining reaction of lipofuscin (Fig 6)

In some cases striking amounts of haemosiderin were present in both xanthoma cells and smaller macrophages (Fig 7) often with a zonal difference

(*Hooper et al* 1962) This is in accordance with the pattern of inflammatory reaction in both human and experimental pyelonephritis (*Heptinstall* 1969) In this connection the tumorous or localized form of XP presents a special problem since urinary tract obstruction seems to be the exception rather than the rule in these cases (*Sel er et al* 1957 *Noyes & Palubinskas* 1969) Microscopically nothing indica tes a morphogenesis basically different from that of the diffuse form and theoretically some sort of intrarenal obstruction might be at work This could not however be substantiated from the present study

In three cases with staghorn pelvic calculi (cases no III V and XVIII) nephrostomy with drainage of pus was done prior to nephrectomy This might explain the disproportionately slight xanthogranulomatous reaction in these cases indirectly pointing to the possible significance of varying degrees of urinary tract obstruction to the quantitative xan thogranulomatous response

Vascular changes mostly in the form of intimal and medial thickening have been observed in a number of cases (*Tonelli & Gianotti* 1969 *Hooper et al* 1962 *Clay et al* 1969 *Schopfer* 1967) The possible significance of ischaemic necrosis has been emphasized by *Habib et al* (1968) in a paper dealing with 4 paediatric cases characterized by an almost selective affection of the renal papillae This would be in agreement with both experimental and clinical observations that increased intrapelvic pressure (due to urinary tract obstruction) and narrowing of vessels (in relation to chronic pyelonephritis or diabetes) seem to favour the development of papillary necroses (*Sheehan & Davis 1969 Heptin stall* 1974) This might explain the high incidence of diabetes mellitus in some series (*Gammill et al* 1975) Papillary necrosis however is probably not the trigger mechanism of xanthogranuloma forma tion since XP so far has not been described in relation to so-called analgesic nephropathy

D

[illegible] the time-correlated structural transforma tion was accompanied by a series of cellular changes indicat [illegible]

dat [illegible]

inte [illegible]

experimental findings and the present observations strongly suggests that they are of basically identical character the only difference being a more incon stant presence and irregular distribution of PAS positive cells This might be due to differences in respect to route of infection or preexistent inflammatory changes in the kidney Consequently it seems justified to believe that the pathogenic mechanisms are similar and rather simple ones with emphasis on urinary tract obstruction This is further illustrated by the findings of *Tan & Heptinstall* (1969) who under similar experimental conditions using however *temporary* occlusion of ureter found an inflammatory reaction with a corresponding time-correlated change in quantity and staining reactions of PAS positive interstitial cells but no formation of typical xanthomatous foci

Ultrastructural investigations have given some evidence that the PAS positive interstitial cells are in some way linked with (ineffective?) bacterial break-down (*Tan & Heptinstall* 1969) As to the xanthoma cells these are not specific to XP but may be present anywhere inflammation and obstruction coexists – »obstructive pneumonia« being the classical example The origin of the fatty substance is disputed Cholesterol esters which make up part of the lipids (*Saeed & Fine* 1963) might derive from lysis of erythrocytes in relation to haemorrhage which applies to the macroscopic appearance and the presence of great amounts of haemosiderin in some cases of the present series

Provided the xanthoma cell and the PAS-positive interstitial cell represent different functional states of the same cell the series of events in the established xanthogranuloma can be explained simply in terms of a normal macrophage response with transformation of intracellular lipids to lipofu scin (*Povysil & Konickova* 1972 *Tan & Heptinstall* 1969)

In conclusion it seems obvious to consider the xanthogranulomatous reaction a post inflammatory resorption phenomenon representing a possible stage in the course of suppurative nephritis (*Tonelli & Gianotti* 1969) As to pathogenesis both the present and previous clinical observations in addi tion to experimental findings point to factors which may increase locally the need for or interfere with disposal of inflammatory waste products As far as urinary tract obstruction haemorrhage in the renal pelvis and focal renal ischaemia represent common denominators in ascending inflammatory kidney disease one might consider the xanthogranuloma tous reaction a matter of concurrence and varying degrees of these factors Considering this and the various morphological expressions of the »disease« and its temporary character (as indicated by both the present observations and the fact that XP is a very rare autopsy finding) it is not surprising to find overt xanthogranulomatous changes only in a minor part of cases of suppurative nephritis

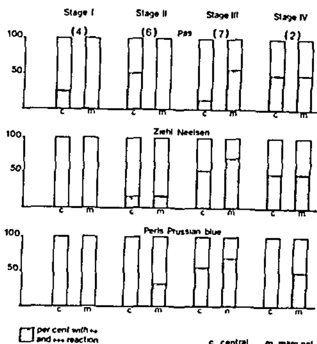


Fig 8 Semiquantitatively estimated staining reactions in central and marginal areas of xanthogranulomas as correlated to morphological stage

ment showed no function on excretory urography, the excretory function being largely inversely proportional to the degree of xanthogranulomatous affection

The type and degree of xanthogranulomatous reaction was found to some extent correlated to the level of urinary tract obstruction, obstructions distal to the renal pelvis being associated with more generalized xanthogranulomatous changes as compared with obstructions above the ureteropelvic junction (Table 3)

TABLE 3 *Quantitative Xanthogranulomatous Changes in Relation to Type and Level of Urinary Tract Obstruction*

	Macroscopic degree of renal involvement		
	I	II	III
Pelvic stone(s) (9)	3	4	2
Uretero pelvic stenosis aberrant vessel (2)	0	0	2
Ureteral stone ureteral carcinoma (6)	1	1	4
Carcinoma of the urinary bladder (1)	0	0	1
No obstructive lesion demonstrated (1)	0	0	1*

* Tumourous form of XP

In one case (no XVIII) partial resection of the kidney was followed half a year later by total nephrectomy In the resected part obvious xanthogranulomatous changes were present In the nephrectomy specimen however, no such changes could be demonstrated

DISCUSSION

In spite of an increasing number of reports on xanthogranulomatous pyelonephritis it is still considered a rare affection (*Chitkara & Singh* 1976) The frequency reported varies considerably depending on the type of material investigated Thus in a series of 3000 consecutive biopsies with *chronic pyelonephritis Malek et al* (1972) found xanthogranulomatous changes in only six per thousand Concerning *pyonephrosis Watt & Roylance* (1975) in a series of 40 cases found XP in almost 25 per cent The fact that one third of the present series (6 cases) was diagnosed prospectively during a relatively short period indicates that the »frequency« of XP to some degree depends on the attention paid to it This too, is illustrated by the series reported by *Hooper et al* (1962)

The clinical appearance, age and sex distribution and bacteriological findings as well as the association of single cases with diabetes pregnancy and obstructive malignant lesions of the urogenital tract are in line with previous observations (*Malek & Elder* 1978, *Hooper et al* 1962, *Friedenberg & Spjut* 1963) and point to a common aetiological and pathogenic basis for XP and pyelonephritis in general

The close connection with pyonephrosis or suppurative nephritis suggests that the xanthogranulomatous reaction is a sequel to local necrosis and abscess formation This is further supported by the observed concordance between different degrees of xanthogranulomatous involvement and the severity of inflammatory kidney disease as indicated by clinical and radiological findings The lack of correlation between the microscopic stage of xanthogranulomatous change and the duration of the clinically acute stage however indicates that the xanthogranulomatous reaction is probably not a direct consequence of tissue break-down but to some extent depends on additional factors

Calculi predominantly of the staghorn type represent a potent obstructive factor in a high percentage of cases (*Chitkara & Singh* 1976 *Malek & Elder* 1978) which is in agreement with the present findings The observation that the xanthogranulomatous changes are to some extent topographically correlated to the level of calculous (and noncalculous) obstruction has been made by others

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DISTRIBUTION OF ATHEROSCLEROSIS IN HUMAN DESCENDING THORACIC AORTA

A Morphometric Study

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Svendsen E & Eide T J Distribution of atherosclerosis in human descending thoracic aorta A morphometric study Acta path microbiol scand Sect A 88 97–101 1980

A morphometric study of 49 randomly selected human aortas is reported with regard to the distribution of atherosclerotic lesions in the descending thoracic aorta (DTA) The intercostal artery area (ICA area) revealed a higher degree of atherosclerosis compared with the remaining part of the DTA The difference was more striking at overall lower grades of atherosclerosis of the DTA The ventral aspect of the DTA in the proximal and caudal thirds showed a higher degree of atherosclerosis compared with the middle third The atherosclerotic indices of the proximal and distal areas correlated significantly with increasing heart weights but not for the middle third The explanation of this is presumably irregular flow conditions For the distal part a possible effect of continuous pounding of an enlarged heart upon the ventral aspect of the descending thoracic aorta is discussed

Key words Atherosclerosis thoracic aorta

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Experimentally induced atherosclerosis in the aorta has shown a focal occurrence (2 21) The sites of occurrence correspond to sites with endothelial cell injury (27 28 29) increased permeability (16) increased platelet deposition (9 11) as well as increased replication (32) and presumed flow disturbance (7 10 18 26) In the literature there are indications that also in the human aorta certain areas are more susceptible to atherosclerotic development (1 3 4 12 20 23) Fatty streaks occur early in the proximity of intercostal artery orifices (23) It is widely accepted that raised blood pressure increases atherosclerotic development and heart weight Alteration of the flow situation may occur (5 13 14) and the enlarged heart gets a closer relation to the ventral aspect of the aorta As all the experimentally recorded phenomena have been demonstrated in the descending thoracic aorta (DTA) a more detailed morphometric study of the atherosclerosis in human DTA seemed appropriate

The following questions were asked

1 Are there differences in the occurrence of atherosclerotic lesions in the intercostal artery area (ICA area) versus the remaining part of the DTA? If so are the differences consistent at different overall degrees of atherosclerosis in the DTA?
2 How is the distribution of atherosclerosis in the non intercostal artery area? Does the heart weight influence this distribution?

MATERIAL AND METHODS

Forty nine aortas all from males were randomly selected from autopsies at the Department of Pathology [illegible]

[illegible] at the dorsal aspect, pinned out on a cork board and fixed in 4% buffered formalin The aortas were slightly dried photographed (dias) and subjected to a

The authors are indebted to the chief pathologists at the departments of pathology at Skive Sygehus Vejle Sygehus Sønderborg Sygehus and Aarhus County Hospital for placing specimens at our disposal

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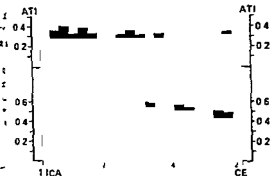


Fig 3 Graphical representation of the atherosclerotic lesions in the non ICA area of the DTA (uppermost diagram) and the ICA area (lower diagram) comprising all cases Each of the twenty-one columns represent the mean atherosclerotic index at the actual level of the aorta 1 ICA – first intercostal artery CE = celiac trunk

middle third however no significant correlation was found In the same figure the mean atherosclerotic indices for each of the non ICA areas are estimated for four groups based on heart weights (200–299 300–399 400–499 and 500 grams and above) The most marked increase of atherosclerosis is observed between the first two groups in all three areas In the group with heart weights above 500 grams there is further marked increase of atherosclerosis in the distal area In the proximal and middle area however the increase is slight

Table 1 shows the mean systolic pressure of the highest blood pressure encountered in the clinical journal of those with heart weight above and below 425 g Mean pulse pressure mean heart weight mean age the number of cases in each group as well as the atherosclerotic indices of the proximal middle and distal thirds of the non ICA area of the DTA are given in the same table The mean systolic pressure and the pulse pressure are somewhat higher in the group with heart weight above 425 g

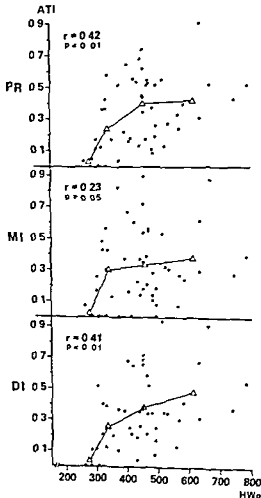


Fig 4 Diagram of the atherosclerotic extent in the cranial middle and distal thirds of the non ICA *area of* the DTA plotted against the corresponding heart weights The triangles represent mean atherosclerotic index within each of the four groups based on heart weights in gram (200–299 300–399 400–499 500 and above) ATI – atherosclerotic index HWg = heart weight in gram r = correlation coefficient PR MI and DI as in Fig 1

DISCUSSION

We present strong evidence that the ICA area is more prone to atherosclerosis than the remaining part of the DTA This finding is in agreement with the work of *Bhagwat et al* (3) The difference is more striking in the groups with overall less atherosclerosis This fact may indicate that the atherosclerotic process starts in the ICA area Experimentally the ICA area is a site of increased permeability (16) platelet deposition (9 11) and endothelial cell injury (27 28 29) Increased cell replication (32) and the inception of experimental atherosclerosis are described in the same area (2 15 21 22) *According* to most authors the particular flow conditions at the ICA area are of importance for the predominance in this area of all the mentioned phenomena

In the non ICA-area of the DTA we found a higher degree of atherosclerosis in the proximal and distal thirds compared to the middle third The degree of atherosclerosis in the non ICA *area of the* proximal and distal parts of the DTA was significantly correlated with the heart weight

morphometric point-counting procedure in which each dias photograph was projected on a screen with regularly arranged points

The reliability of the morphometric method had been controlled in a study comprising 22 aortas Intra- and inter observer comparisons showed a high reproducibility by using statistical tests The correlation coefficient ranged from 0 96 to 0 98 in both inter- and intra observer comparisons The standard deviation of the random measurements error expressed in per cent of the mean value of the replicate measurements ranged from 8 to 12 per cent, and the slope of the regression coefficient did not differ from unity by more than 0 045 in any of the comparisons (6)

By adjusting the distance from the dias-projector to the screen each DTA of all 49 aortas comprised a standardized number of 21 point-rows from the I intercostal artery orifices to the celiac trunk All points falling upon fatty streaks, plaques and complicated lesions (ulcerations, hemorrhage, calcifications and mural thrombus) were counted as atherosclerotic lesions and mapped on graph paper together with points falling upon normal areas The DTA was divided into three equal fields each comprising seven point-rows and was also divided into ICA-area and non intercostal artery area (non-ICA-area) as shown in Fig 1 The points falling closest lateral to the intercostal artery orifices and points falling at the dorsal wall in between the intercostal artery orifices were allotted to the ICA area All oth points falling upon the DTA were allotted to the no ICA-area which represented the lateral and ventral aspe of the DTA A mean atherosclerotic index for the who ICA-area and the whole non ICA-area was estimate Also a mean atherosclerotic index for each of the 2 point-rows in the ICA-area and non ICA area of all 4 aortas was calculated Furthermore mean atherosclerot indices of the proximal, middle and caudal third of t non ICA-area of the DTA were estimated A correlatic analysis between the heart weights and atherosclerosis i each of the latter-mentioned areas was made

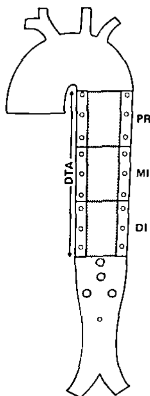


Fig 1 Schematic representation of the opened aorta DTA = descending thoracic aorta, PR = proximal part of DTA, MI = middle part of DTA, DI = distal part of DTA The hatched field represents the non ICA-area of the DTA, the unhatched field of the DTA the ICA area

RESULTS

In the descending thoracic aorta the ICA are showed consistently relatively more atheroscleros than the non-ICA area The difference between th two areas was greatest in the groups with low tot atherosclerosis and less in the groups with high tot atherosclerosis (Fig 2)

A mean atherosclerotic index based upon all 4 aortas for each of the 21 point-rows in the ICA are and non-ICA-area of the DTA was calculated I the non-ICA-area more atherosclerotic lesions wer found in the proximal and distal thirds compare with the middle third (Fig 3) In the same area caudal increase of atherosclerosis at the ver beginning of the DTA and a decrease whe approaching the celiac trunk was observed (Fig 3) In the ICA-area, however, the distribution had a opposite pattern (Fig 3)

Fig 4 shows the heart weights plotted against th respective atherosclerotic indices for the proximal middle and distal thirds of the non-ICA-area of th DTA In both the proximal and distal thirds ther are statistically significant correlations betwee heart weight and degree of atherosclerosis In th

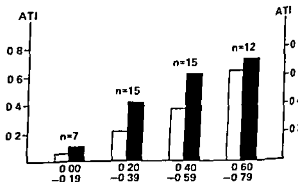


Fig 2 Extent of atherosclerotic lesions in the ICA area (filled bars) versus non ICA-area of the DTA (open bars) Four groups based on increasing overall atherosclerotic index of the DTA are arranged The bars express mean values ATI = atherosclerotic index

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TABLE 1 *Clinical Parameters, Mean Heart Weights as well as Atherosclerotic Indices of the Non-Intercostal Artery Area of the Descending Thoracic Aorta in Groups with Heart Weight above and below 425 g*

		Heart weight ≧ 425 g	Heart weight < 425 g
Number		29	20
Mean age (years)		68 2	64 4
Mean heart weight (g)		518	340
Mean systolic BP (mmHg)		167 6	150 5
Mean PP (mmHg)		66 8	62 3
Mean ATI of non-intercostal artery area of the descending thoracic aorta	Proximal third	0 41	0 22
	Middle third	0 34	0 27
	Distal third	0 40	0 27

BP = blood pressure PP = pulse pressure ATI = atherosclerotic index

Knierem & Hueber (12) found a diffuse, in some cases, patchy thickening of the aortic intima, especially of the distal thoracic aorta, with increasing age, and the weight of the aorta increased with increasing heart weight

The preponderance of atherosclerosis in the proximal third of the non-ICA-area compared to the middle third of the DTA may be explained by the occurrence of boundary layer separation with vortices This area blends with the lesser curvature of the aortic arch where this type of flow disturbance is supposed to occur (7, 10, 15 26) Hypertension may leave the cardiac output unaltered (8) Labile hypertension, however, is attended by increased cardiac output (5, 14) Further reduced *distensibility of large arteries (19, 31) and increased* pulse wave velocity (32) may occur in hypertension Thus, raised blood pressure may produce a more intensive flow disturbance in the distal area of the lesser curvature of the aortic arch

The preponderance of atherosclerosis in the distal third compared with the middle third of the non-ICA-area of the DTA may also have a similar explanation This site however, has hitherto not been suspected to have more flow disturbance than the middle third Hydrostatic pressure may play a role The fact however that the atherosclerotic process decreases when approaching the celiac trunk may be an argument against this assumption If we can take it for granted that the hearts with more than normal weight have a larger than normal size, an alternative explanation may be that the increased presence of atherosclerosis in the non-ICA-area of the distal third of the DTA is a direct effect of the enlarged heart The enlarged heart is located closer to the ventral aspect of the aorta It is possible that the beats of an enlarged heart may pound against aorta to a greater extent than normal and thus af the aorta This explanation is in accordance w *Solberg & Eggen* (24) as well as *Palma* (17) *Stephanson et al* (25) who put forward hypothesis that atherosclerosis may occur as a re of mechanical influence on the vessel wall fr outside

In conclusion, this work has shown a consist predominance of atherosclerosis in the ICA a versus the non-ICA-area of the DTA at all degr of atherosclerosis

This predominance is more pronounced patients with a small or moderate overall deg atherosclerosis of the DTA

Atherosclerosis is more prevalent in the upp and lower thirds of the non-ICA-area of the DT compared with the middle third The two forme mentioned areas in contrast with the latter correla significantly with increasing heart weight

We owe our gratitude to Prof *L Jørgensen* and Dr *L Solberg* for professional critism Dr *Alex Tindall* f reading and Mrs *Dagny Madsen Fauske* for typing t manuscript

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IMMUNOCYTOCHEMICAL DEMONSTRATION OF ENKEPHALIN AND β-ENDORPHIN IN ENDOCRINE TUMORS OF THE RECTUM

A Survey of 27 Colo Rectal Carcinoids

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Alumets J Falkmer S Grimelius L Håkanson R Ljungberg O Sundler F & Wilander E Immunocytochemical demonstration of enkephalin and β-endorphin in endocrine tumors of the rectum A survey of 27 colo rectal carcinoids Acta path microbiol scand Sect. A 88 103–109 1980

In a histopathological and immunocytochemical study of biopsy and/or operation specimens from 27 patients with endocrine tumors of the colon and rectum (hind gut carcinoids«) enkephalin immunoreactive tumor cells were observed in two cases Both patients were obese women about 50 years of age with a history of constipation The tumors were situated near the anus in the dorsal wall of the rectum One tumor had metastasized to a lymph node and the other showed vascular invasion The tumor cells were non argentaffin some were argyrophil One tumor contained only few enkephalin immunoreactive cells but had numerous β-endorphin immunoreactive cells which were distinct from the former The other contained large numbers of enkephalin immunoreactive cells but no β-endorphin cells Both tumors also harboured glucagon immunoreactive cells in one there were also cells containing immunoreactive pancreatic polypeptide These cells were distinct from the enkephalin-storing ones No 5 hydroxytryptamine could be detected in the two tumors

Key words Rectal carcinoid mixed endocrine tumor enkephalin β-endorphin immunocytochemistry

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Accepted as submitted 16 x 79

The pentapeptide enkephalin first isolated from brain tissue by virtue of its potent opiate receptor agonist activity (1) occurs in two varieties one having leucine (»leu-enkephalin«) the other methionine (»met-enkephalin«) in COOH terminal position (1) By immunocytochemistry enkephalin like material has been demonstrated in nerve fibres and nerve cell bodies of the gut wall and in endocrine cells of the antrum and duodenum (2 3) The endocrine cells demonstrated with antiserum against leu-enkephalin were found to be of the 5 hydroxytryptamine (5 HT)-containing type (3) Enkephalin immunoreactive cells have recently been demonstrated also in the exocrine parenchyma of pig pancreas (4) and in some bronchial endocrine tumors (5) Another endogenous *opioid peptide is β*-endorphin which occurs in high concentrations in the pituitary gland including pituitary adenomas (6) So far β-endorphin has not been found in peripheral tumors

The findings of immunoreactive enkephalin in gut endocrine cells and in bronchial carcinoids prompted a search for enkephalin in endocrine tumors of the digestive tract We started by examining 27 cases of colo-rectal carcinoids*) In all two cases were found They form the basis of the present report

*) The term »carcinoid« is used here to mean primary endocrine tumors of the digestive tract irrespective of the presence or absence of 5 HT (7)



Fig. 1. *Case No. 1.* Low and medium power photomicrographs of hematoxylin-erythrosin stained sections of the rectal carcinoid and adjacent mucosal crypts showing nests and trabecules of small regular epithelial tumor cells growing in the basal part of *lamina propria* invading the submucosa (Fig. 1A) and displaying the ribbon growth pattern typical of this kind of tumors (Fig. 1B). By the PAP immunocytochemical procedure cells were found displaying β-endorphin immunoreactivity in the normal mucosa overlying the tumor (Fig. 1C arrows) as well as in the tumor itself (not shown in the figure) whereas enkephalin immunoreactivity occurred in a few scattered tumor cells only (Fig. 1D). X100(1A), X255(1B), and X300(1C and D).

MATERIAL AND METHODS

The material consisted of blocks of paraffin embedded specimens from hind gut carcinoids collected from 27 patients Twelve of these tumors had previously been analyzed for the presence of cells containing glucagon and substance P like immunoreactivity (8) The specimens were either from extirpated tumors or from biopsy specimens they were fixed in 10% formalin For light microscopic analysis 4–6 µm thick sections were stained with haematoxylin eosin van Gieson s stain the periodic acid Schiff (PAS) reagent the Masson Hamperl argentaffin technique (9 10) as well as with the Grimelius argyrophil procedure (11)

Deparaffinized sections from all tumors were examined for enkephalin and β endorphin immunoreactive cells using the indirect immunofluorescence method (12) or the peroxidase anti peroxidase (PAP) procedure (13) Sections were thoroughly rinsed in buffer over night and incubated with the antiserum for 3 hrs at room temperature (immunofluorescence) or for 24 hrs at + 4 °C (PAP procedure) The sections were washed repeatedly with phosphate buffered saline and the site of the antigen antibody reaction was revealed by incubation with fluoresceinated or non fluoresceinated anti rabbit IgG in dilution 1 20 In the PAP procedure the PAP complex was applied in dilution 1 320 Control sections were exposed to antiserum inactivated by the addition of antigen in excess (100 µg/ml) The tumors displaying opioid peptide immunoreactivity were then analyzed histochemically for various other hormones and hormone candidates Details of the antisera used are given in Table 1 Sections from these tumors were also examined for 5 HT This amine condenses with formaldehyde yielding a fluorophore which is detectable upon excitation with UV light (365 nm) (32)

RESULTS

Two out of the 27 examined rectal carcinoids contained enkephalin immunoreactive cells (case 1 and 2 below) In addition one of the two (case 1) contained β endorphin immunoreactive cells

Case No 1 Previously healthy obese 49 year-old woman was examined by rectoscopy because of pains in the sacral and anal regions For several months she had suffered from constipation A slightly protruding nodule about 1 cm in diameter was observed located on the dorsal wall 5–7 cm above the anus An X ray examination of the colon showed no other nodules polyps or tumors A biopsy specimen was taken The histopathological examination (Umeå 25932/76) revealed a rectal carcinoid About a month later an abdominal perineal extirpation of the rectum was made with a sigmoideostomy About half the tumor remained in the extirpated rectum It was a firm nodule with an

TABLE 1 *Survey of Antisera Used*

Antisera raised against	Code No	Working dilution Immuno fluorescence	PAP staining	Source	Refere
Adrenocorticotropic hormone (ACTH)		1 80	1 640	Own	14
β Endorphin	7763	1 20	1 240	Milab Malmo Sweden	15
Leu Enkephalin	170	1 60	1 240	K J Chang Burroughs Wellcome Research Triangle Pk N C USA	3 16
Gastric inhibitory peptide (GIP)	R D 11/19/77	1 80		T O Dorisio Ohio State University Columbus Ohio USA	17 18
Gastrin (2 17)	4562	1 640	1 25000	J F Rehfeld Århus Univ Århus DK*)	19
Glucagon	4304 XII	1 80	1 2560	J J Holst Bispebjerg Hosp DK	20 21
Human pancreatic polypeptide (HPP)		1 20		R E Chance Eli Lilly Indianapolis Indiana USA	22
Insulin	LA 1	1 80		L Heding Novo Res Institute DK	
Motilin	MBR01 6		1 640	N Yanaihara Shizuoka Coll Pharm Jap	23
Neurotensin	HC 8	1 40	1 640	R E Carraway Harvard Med School USA	24 25
Secretin	5585	1 80	1 2560	J Fahrenkrug & O B Schaffalitzky de Muckadell Bispebjerg Hosp DK	26 27
Somatostatin	19578	1 80	1 1280	M P Dubois Inst Nat Recherche Agriculture Nouzilly France	28 29
Substance P	JKI	1 320		P Emson Cambridge University GB	
Vasoactive intestinal polypeptide (VIP)	89 N		1 320	S I Said Dallas Tx USA	30 31

DK Denmark

apparently intact overlying mucosa Its cut surface was grey white and it grew mainly in the submucosa and the

constipation and no signs of the carcinoid syndrome when examined three years after the operation (in January 1980)

The tumor appeared well differentiated and was composed of small uniform polyhedric cells with unimorphic nuclei arranged in thin trabecules which often were only one cell in thickness and which formed a marked whorled ribbon growth pattern (Fig 1) There were no PAS positive cells Mitoses were inconspicuous A few cells displaying leu-enkephalin like immunoreactivity were found scattered in the tumor tissue Fairly numerous tumor cells contained immunoreactive β-endorph n Such cells were also found in the mucosa verlying the tumor Many tumor cells also displayed moderate to intense glucagon immunoreactivity No tumor cells showed formaldehyde induced fluorescence The enkephalin immunoreactive cells were weakly argyrophil no tumor cells were argentaffin This tumor did not belong to the previously published series (8)

Case No 2 The patient was a previously healthy obese 53 year-old woman suffering from constipation Rectal palpation and rectoscopy revealed a large bulging tumor situated in the dorsal wall of the rectum just above the anal sphincter The overlying mucosa was intact but the tumor appeared rather firmly attached to the coccyx A biopsy specimen was taken The microscopic examination suggested a poorly differentiated carcinoid X ray examination of the colon and rectum revealed an expansive process between the rectum and the sacrum there were no signs of additional tumors A pre-operative high voltage X ray therapy of altogether 3 000 rad given for 3 weeks had no effect on the tumor mass An abdomino perineal rectum extirpation and colostomy was made The tumor seemed to have invaded the periosteum of the sacrum but could be radically extirpated The gross and microscopic examinations (Halmstad 3017/78) showed a large solid tumor just above the anus measuring 5 × 10 × 15 cm with an intact rectal mucosa The cut surface was pink with necrotic areas in the central parts The tumor was encapsulated. The postoperative course was complicated by anuria requiring catheterization of the ureters b lateral nephrostomy and haemodialysis At the latest follow up (Febr 1980) almost two years after the operation the patient had no constipation and there were no signs of the carcinoid syndrome

The tumor had a lobular architecture forming nodules of irregularly arranged and closely packed tumor cells surrounded by broad strands of connective tissue stroma The cells displayed a marked anaplasia with irregular atypical nuclei showing coarse chromatin pattern with plump nucleoli and mitoses were frequent (Fig 2) In some places there was a gradual transition towards a more differentiated tumor where a distinct ribbon growth pattern could be found which was quite similar to that seen in the tumor in case No 1 There were no PAS-positive tumor cells The tumor had infiltrated through all layers of the bowel wall into the perirectal and perianal tissue and veins were also invaded Certain areas contained numerous tumor cells displaying moderate to intense leu-enkephalin like immunoreactivity whereas other areas were devoid of such cells No enkephalin immunoreactive cells were observed in the overlying mucosa In addition many tumor cells were found to contain immunoreactive glucagon or HPP Immunocytochemical staining of consecutive sections revealed that the enkephalin storing cells were distinct from these two cell types No β-endorphin cells could be demonstrated Most tumor cells were argyrophil none were argentaffin No cells displayed yellow formaldehyde induced fluorescence indicating absence of 5 HT Even this tumor was not included in the previously published series (8)

DISCUSSION

The two patients whose rectal carcinoid tumors contained enkephalin immunoreactive cells had the following features in common They were both obese women about 50 years of age with a history of constipation Both tumors were located near the anus in the dorsal wall of the rectum The overlying mucosa was intact, but both tumors were obviously malignant in one case with a lymph node metastasis Both tumors also contained well differentiated areas with the features of hind-gut carcinoids (7) In case No 2 the major part of the

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cells exhibiting enkephalin like immunoreactivity have to the best of our knowledge not been described in endocrine tumors Recently we have observed immunoreactive enkephalin in scattered cells of a pancreatic islet-cell tumor This tumor contained also numerous vasoactive intestinal peptide (VIP) immunoreactive cells and was associated with watery diarrhoea By personal communication we have been informed that enkephalin immunoreactive cells have been observed in a pancreatic endocrine tumor also by others (33)

Gastro-entero-pancreatic tumors often contain several different peptide hormone producing cell types Nevertheless many such tumors are clinically »silent« and are often found incidentally at autopsy Others give rise to clinical symptoms which as a rule reflect overproduction of only one such hormone (34) Although enkephalin inhibits gut neuronal activity and β-endorphin has been associated with overeating (35) we believe that the modest clinical symptoms displayed by the two

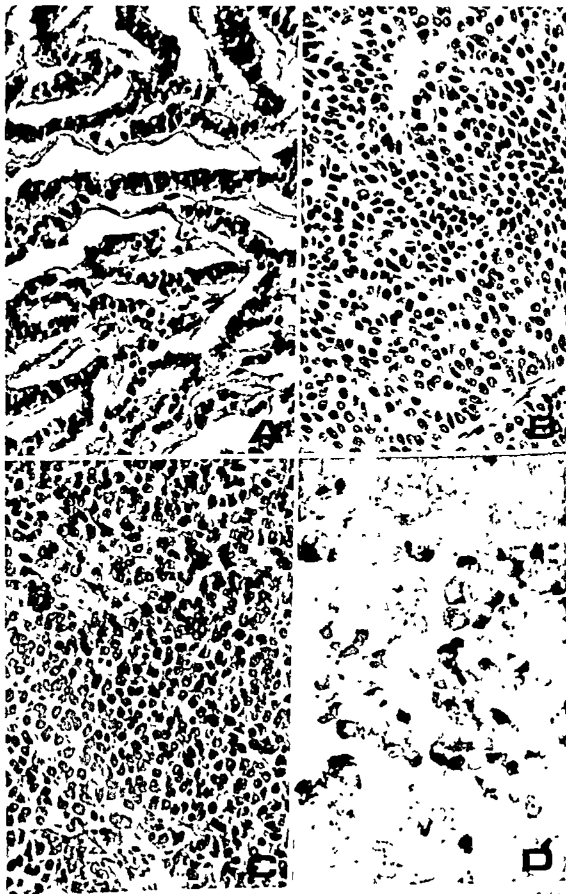


Fig 2 – Case No 2 Medium power photomicrographs of hematoxylin-erythrosin stained sections of the rectal carcinoid showing the wide range of differentiation observed in various parts of the tumor Areas of the same ribbon growth pattern as in the tumor of Case No 1 occurred (Fig 2A) merging into moderately well differentiated parts with rosette formations and palissading cells in the margin of tumor nodules (Fig 2B) In the most undifferentiated parts only closely packed irregularly distributed tumor cells with marked atypia were found (Fig 2C) By the PAP immunocytochemical procedure fairly numerous tumor cells were displaying enkephalin immunoreactivity of varying intensity (Fig 2D) The cells occurred as single cells or as clusters X255(2A, B X300(2D)

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patients do not necessarily reflect overproduction of endogenous opiates

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EFFECT OF ESTRADIOL AND PROGESTERONE ON THE ADENYLATE CYCLASE ACTIVITY IN GRAFTS OF 3-METHYLCHOLANTHRENE INDUCED CARCINOMAS IN THE MOUSE UTERINE CERVIX

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Kvinnsland S Lønning P E & Forsberg J G Effect of estradiol and progesterone on the adenylate cyclase activity in grafts of 3 methylcholanthrene induced carcinomas in the mouse uterine cervix Acta path microbiol scand Sect. A 88 111-118 1980

Grafts from a methylcholanthrene induced mouse uterine cervical carcinoma (NMRI mice) were grown in neonatal female mouse hosts The hosts were treated for 12 days with estradiol and progesterone alone or in combination Controls were injected with the solvent (olive oil) only Pieces from every graft were used for histological study for study of the proliferation rate and for study of the adenylate cyclase activity in homogenate All the grafts were well differentiated squamous carcinomas and the histological picture was the same irrespective of hormonal treatment The proliferation rate was the same in control grafts as in those from hosts treated with hormones Grafts from progesterone and progesterone/estradiol injected hosts had significantly higher levels of adenylate cyclase activity compared with grafts from control hosts Estradiol alone resulted in a trend towards increased activity but the difference to controls was not statistically significant

Key words Cervical carcinoma mouse neoplasm transplantation adenylate cyclase estradiol progesterone

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The effect of exogenous sex steroid hormones on the incidence and latency period of chemically induced uterine cervical carcinomas in mice has been extensively studied often with highly contra dictory results (for a review see 32) For the present study it is enough to mention that ovariectomy may be associated with an increased incidence of invasive tumors while intact females have a higher incidence of *in situ* carcinoma Estrogen may inhibit invasiveness (26 27 30) possibly through an effect at the differentiation level (22 33) The histological type of tumour may be dependent on hormonal conditions ovariectomy and progestins promote mucin-secreting or mucoepidermoid tumours while estrogen promotes squamous cell carcinomas (17, 36)

Manifest tumours of different types are reported to have an increased basal activity of adenylate cyclase and changed sensitivity to hormones or PGE_{1+2}(4 19 20 24 28 34 37) However there is a wide variation in tumour adenylate cyclase response to hormones and other factors (12) A decreased intracellular cAMP level has been descri bed for e g colon adenocarcinoma and from tumour cells or transformed cells grown *in vitro* (11 13 40 42)

As both cytochemical and biochemical studies indicate an effect of exogenous estradiol on the adenylate cyclase activity in the neonatal normal

Calif) draining into a neutral alumina column *(Bio Rad)*. The eluate (4 ml) from the alumina column was collected into a scintillation vial 16 ml of Instagel *(Packard)* was added and the radioactivity counted in a *Packard* 2425 Tri-Carb liquid scintillator with separate channels for ^{3}H and ^{32}P. The recovery of product was 50–60%. Control incubations containing boiled homogenate or buffer were incubated in each series.

Adenylate cyclase activity in homogenates from 15 tumours (including tumours form olive oil and hormonal treated animals) was estimated from linear parts of curves relating enzyme activity either to incubation time or protein concentration. Adenylate cyclase activity was linear with incubation time for at least 10 min and with protein concentration between 50 and 300 μg per assay in the presence and absence of NaF. Enzyme activity of the other homogenates was measured in triplicate incubation time 10 min and protein concentration 200–250 μg. The results were exoressed as pmoles cAMP produced per 10 min and related to mg wet weight, mg protein or mg DNA.

Measurement of DNA and protein in homogenates. Part of the homogenate from every tumour was dissolved in 10 volumes of 1 N NaOH and the DNA concentration measured by the method described above using 3 dilutions (each measured in duplicate) from each homogenate.

Protein was measured in the dissolved homogenate using the method of *Klungsøyr* (23) with bovine serum albumin *(Sigma)* as standard. Protein concentration was measured in 3 dilutions from each homogenate.

Statistical method. Provided the error variance between different groups to be compared did not vary signif[illegible]
of va[illegible]
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measurements (41). The T values from the *Mann Whitney* test and significance levels are given in Figs 2–5. Significance levels $0.05 > p > 0.01^{*}$ (probably significant) $0.01 > p > 0.001^{**}$ (significant).

RESULTS

Histology. Histological sections of biopsy pieces from the same grafts as used for studies of ^{3}H TdR incorporation and adenylate cyclase activity all showed well growing cancer tissue without any round cell infiltration (Fig 1). Irrespective of the sections being taken from grafts in control or hormonally treated females (estradiol and/or progesteron) the tumour tissue had the same histological type well-differentiated squamous carcinoma. The histological study did not reveal any tendency to a mucoepidermoid tumor type in grafts exposed to estradiol and progesterone in combination or progesterone alone. The distribution between the stromal and epithelial compartment did not seemingly differ between the different grafts studied.

^{3}H-TdR incorporation. The results are demonstrated in Fig 2. None of the hormonal treatments used (E_2, E_2 + P, P) influenced the proliferative activity as measured by the ^{3}H TdR incorporation per μg DNA. Even though the variances seemed to be larger in the E_2 + P and P groups compared with the control and E_2 groups a *Bartlett's* test for homogeneity of variances did not demonstrate any significant difference in intragroup error variances.

Adenylate cyclase activity. The adenylate cyclase activity, measured as the formation of cAMP during a 10 min period was related to the wet weight of the preparations studied (Fig 3) and to their DNA (Fig 4) and protein (Fig 5) content all homogenates were incubated in the absence or presence of NaF grafts from E_2 treated hosts had higher mean values for enzyme activity than preparations from olive oil injected hosts but the difference was never statistically significant. However irrespective of the enzyme activity being related to wet weight DNA or protein content grafts from E_2 + P and P treated hosts had statistically significant higher enzyme activity than controls and this both in the absence or presence of NaF in the incubation medium. In no case was there any difference between the E_2 + P and P groups.

When the adenylate cyclase activity per mg DNA (Fig 3) was compared in homogenates without or supplemente with NaF, it was evident that the mean quotient (+/– NaF) was 3.15 for control grafts 2.85 for grafts from E_2 injected hosts and 2.58 and 2.70 for grafts from E_2 + P and P treated hosts respectively. A variance analysis of these quotients demonstrated probably significant higher quotients for the control grafts than for the hormonally treated grafts ($F = 3.127$ for 3 and 25 d.f. $0.05 > p > 0.01$). Similar results were obtained also when the enzyme activity was related to the protein content or wet weight.

No correlation occurred between ^{3}H TdR incorporation and adenylate cyclase activity neither in control nor in hormonally stimulated grafts.

DISCUSSION

All the tumour grafts included in this study belonged to the 10th transplantation generation of one single methylcholanthrene induced primary cervical carcinoma. Both the primary tumour and the transplantation generation was randomly chosen for this study among all the transplantation generations from different primary tumours available at the laboratory. Moreover, because of the transplantation protocol all the grafts in this material are descended from one single graft in the

uterine cervical epithelium of mice (25), we found it of interest to study the adenylate cyclase response to exogenous estradiol and progesterone in grafts of methylcholanthrene induced mouse cervical carcinomas

MATERIAL AND METHODS

Animals All the animals used belonged to a non inbred NMRI mouse strain The animals were fed a standard pellet diet and given water *ad libitum* A few days before birth pregnant females were put into separate cages (one female in every cage) where they gave birth to their young On the day of birth the male and female young were separated and every mother allowed to nurse 8–10 females

Tumour induction Female mice 6–9 weeks old were laparatomized under ether anaesthesia A cotton thread impregnated with 3 methylcholanthrene (MCA Sigma) in beeswax was inserted into the uterine cervix as earlier described (15 31) Beginning 3 months after insertion of the MCA thread and thereafter once every other week the females were palpated and when a palpable cervical tumour was identified the female was killed The tumour was dissected out and put into *Parker* medium 199 Part of the tumour was placed in *Bouin s* solution for fixation and later histological study The remaining part was cut into small pieces and washed twice in *Parker* medium 199 with added penicillin and strepto mycin (penicillin 100 IU/ml streptomycin 100 μg/ml)

Tumour grafting Neonatal females were used within 24 hours after birth They were anaesthetized by cooling on ice A small cutaneous incision was made on the left side of the abdomen and from this incision two subcutaneous »tunnels« were made with a small forceps Two small tumour pieces were placed in each tunnel and the skin incision closed with 8/10 thread on an atraumatic needle

About two weeks (11–18 days) after transplantation the hosts were checked for tumour growth The size of the transplants varied but on every host it was possible to find one or two tumour grafts showing active growth with a several fold enlargement compared with the inserted tumour pieces

Those grafts with an active growth were removed and placed in *Parker* medium 199 Most of the tumour tissue was frozen and stored in liquid nitrogen Before freezing small parts were removed from different regions of the tumour graft and these pieces were used for biopsy One of the pieces was cut into smaller pieces and these were used for re transplantation into new hosts according to the same technique as described above In this way tumour pieces from the same primary tumour were re transplanted at an interval of about two weeks From some primary tumours we have now transplanted for 15 generations

Hormonal treatment Nineteen hosts carrying the 10th generation of transplants from a single primary tumour were used Beginning on day 3 after birth the host females were given subcutaneous injections of 0 05 ml olive oil (controls 6 females) 1 μg estradiol 17β (E_2 7 females) 500 μg progesterone (P 3 females) or combination of E_2 and P (3 females) The hormon[illegible] were dissolved in olive oil and were both from *Sigm[illegible]* The host females were injected with single dai[illegible] injections for 12 days and killed 24 hours after the la[illegible] injection One or two well growing tumour transplan[illegible] were removed from every host for further study

Studies on ^3H-thymidine (^3H-TdR) incorporation From every tumour transplant removed 9 small piece[illegible] were randomly cut out from different regions of th[illegible] tumour, while the rest of the tumour was preserved [illegible] liquid nitrogen for further treatment (see below) Th[illegible] pieces were incubated in *Leighton* tubes (3 pieces in each tube) in 2 ml medium (90% *Parker* medium 199 10% fetal calf serum/*Gibco*) supplemented with 5 μCi ^3H TdR (sp act 5 Ci/mmol *Radiochemical Centre Amersham*) The incubation took place in an atmosphere of 95% O_2 and 5% CO_2 (non-capped tubes) at 37 °C for 1 hour After incubation the tumour pieces were washed in 5% trichloroacetic acid (4 °C) for 1 hour (3 shifts) and finally for 15 min in saline The preparations were solubilized in 1 N NaOH and the DNA content was measured using the indole method (2) *Calf thymus DNA (BDH Biochemicals)* was used as standard To 100 μl of the NaOH hydrolysate was added 1 ml *Luma Solv[illegible]* (*Lumac Systems AG* Basel) and 14 ml scintillation liquid (fluorochromes in toluene) Scintillation counting took place in a *Packard* 2425 Tri Carb liquid scintillator The counting efficiency was 36% The radioactivity was expressed as cpm/ μg DNA and this value was taken as a measure of cell proliferation

Preparation of homogenates The tumours were stored in liquid nitrogen weighed on a sensitive *Metzler* balance and homogenized (*Thomas type A teflon/glass* homoge nizer 975 rpm 5 strokes) in 10 volumes of 2 mM glycylglycine buffer (pH 7 4) containing 20 mM KCl 20 mM NaCl and 1 mM $MgSO_4$ The homogenate kept on ice was assayed for adenylate cyclase activity within 30 min

Adenylate cyclase activity The method described by *Salomon et al* (39) was used with minor modifications An aliquot (50 μl) of the homogenate was incubated in a total volume of 100 μl containing 25 mM Tris HCl buffer (pH 7 6) 5 mM $MgCl_2$ 1 mM adenosine 3 5 cyclic monophosphoric acid (cAMP *Sigma*) 20 mM phosphocreatine (*Sigma Chem Co*) 100 U/ml creatine phosphokinase (Sigma Chem Co) and 1 mM ATP (*Sigma Chem Co*) 2–4 × 10^6 cpm –^{32}P ATP (sp act 15–25 Ci/mmol *The Radiochemical Centre Amersham*) and in some experiments 5 mM NaF The reaction was started with the addition of the homogenate and was [illegible] 37 °C The enzyme [illegible] 975 [illegible] mple was heated in a boiling water bath for 4 min The samples were then centrifuged at 1200 g for 10 min To 900 μl of the supernatant was added ^3H cAMP (ca 10 000 cpm sp act 27 Ci/mmol *The Radiochemical Centre Amersham*) in 100 μl Tris HCl buffer and the 1 ml sample was applied to a *Dowex* column (AG 50W X4 200–400 mesh H^+ form *Bio Rad Richmond*

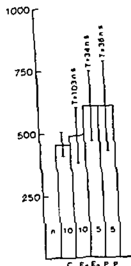
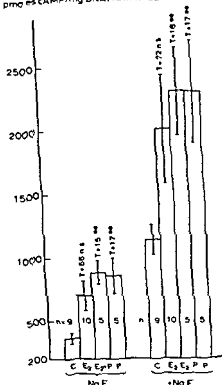
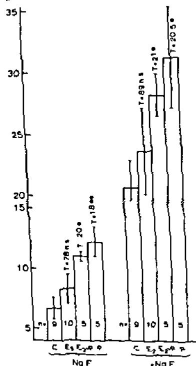


Fig 2 The cell proliferation (^{3}H TdR incorporation into DNA) level in grafts from differently treated hosts C controls E_2 estradiol P progesterone





Figs 3–5 The adenylate cyclase activity in grafts from differently treated hosts (C controls E_2 estradiol P progesterone) and in the absence or presence of NaF The enzyme activity was related to mg wet weight (Fig 3) mg DNA (Fig 4) or mg protein (Fig 5) For T values and significance levels see Statistical method

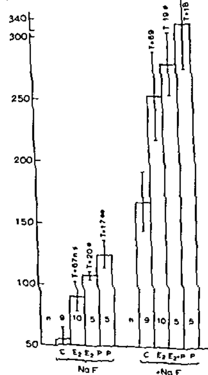




Fig 1 A–D Microphotographs of sections of grafts from control *(A)* or hormone treated hosts *(B–D)* *B* estradi
C estradiol and progesterone *D* progesterone Magnification 220 ×

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9th transplantation generation This confers a high degree of cellular homogeneity on the material used, which is also visible from the histological study of biopsy pieces from the grafts The grafts displayed an active growth in the neonatal hosts, without any evidence of round cell infiltration or necrosis

All the female hosts were killed on day 15 after birth, after being injected with hormone(s) or olive oil on day 3–14 Day 15 was chosen for sacrifice because all our previous results showed that at this time surviving grafts are in a period of active growth The duration of the hormonal treatment was rather long because we were more interested in long-term results than in »acute« effects

It has earlier been pointed out that there is an inconsistency in the results when the level of cyclic nucleotides or activities or related enzymes are expressed relative to protein, RNA, DNA, wet weight or cellularity (24, 29, 37) In this study the adenylate cyclase activity was related to wet weight, mg protein and DNA content, and in all three instances there was a remarkable degree of consistency

The most important results from this study are the definite evidences for a steroid-induced influence on the adenylate cyclase activity, and this in a tumour type where steroid effects have been debated So far the results are in agreement with those earlier described (25) from experiments using neonatal mouse cervical preparations The general opinion in literature is that the adenylate cyclase – cAMP and its effector system are not involved in the mechanism of steroid action (43)

Some reports, however, stress that steroids may have important effects related to the cell membrane (1, 35) The mechanism behind the steroid-induced activation of the adenylate cyclase in neonatal mouse uterine cervix and cervical tumours is not known Both direct and indirect effects are possible (21, 38) The NaF induced adenylate cyclase activation was less pronounced in grafts from hormonally treated hosts compared with controls

Treatment of the hosts with estradiol and/or progesterone did not influence the cellular proliferation in the grafts as measured from ^{3}H-TdR incorporation per mg DNA However an earlier and transient estradiol induced proliferation wave should not be excluded from these results Nor in our earlier studies have we obtained evidence for estradiol alone stimulating the growth of primary cervical tumours (16) The failure in this study to find any hormonally induced effects on cell proliferation argues against the possibility that the increased adenylate cyclase activity in the grafts, after injecting the hosts with progesterone, is related to a hormone induced tumour regression (7, 18)

Generally speaking, increased levels of cAMP l a growth-arresting effect on cells in vitro (5 6 and exogenous cAMP can induce regressio responsive Walker 256 mammary carcinoma (11) Rat mammary carcinomas, induced with dimethyl-4-amino biphenyl, are reported to ha increased activity of adenylate cyclase as comp with the normal, lactating mammary gland (3) same holds true for the basal adenylate cy activity in different other types of tumours (4 20, 24, 28, 34, 37)

No conclusions should be drawn from increased enzyme activity described in this p about the actual intracellular level of cAMP latter reflects both synthesizing and degra processes, only the former is reported Increased cAMP-phosphodiesterase activity ma concomitant with increased adenylate cyclase vity (8) Moreover, an increased level of cAMP be combined with mutational changes in the cA effector system, leading to a defect protein kinas 12, 19)

All the tumour grafts, irrespective of treatme the hosts, were well-differentiated squamous carcinomas Neither progesterone nor progeste in combination with estradiol resulted in tendency towards a mucinous or mucoepiderm tumour type This is in contrast to results repo earlier both from this (16) and other laborato (36) As the latter results are based on treatmen animals with primary tumours for longer pe than used in this study, the discrepance may explained by bothe the time factor and spe characteristics of the primary tumour used here

The consequences of the steroid induced act tion of the adenylate cyclase in the grafts can onl a matter of speculation before other links in cAMP-effector system have been studied Stud on 12 primary methylcholanthrene-induced cerv carcinomas revealed no differences in protein kin properties compared to normal vaginal epitheli (4) It should not be excluded that both the neon mouse cervix and the tumour grafts studied h reveal in a more analysable way an aspect of steroid mechanism of action which is difficult grasp in normal immature and adult stages

This investigation was supported by grants from Norwegian Cancer Society

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FORMATION OF LARGE VACUOLES IN TUMOUR CELLS GROWN IN SUSPENSION

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Jakobsen A L & Chemnitz J Formation of large vacuoles in tumour cells grown in suspension Acta path microbiol scand Sect A 88 119-124 1980

In suspension cultures of JB 1 E cells approximately 11% of the cells contained a single large vacuole (balloon cells) or signet ring cells) Such cells were rarely observed in monolayer cultures where the cells were attached to a substrate Only a few »balloon cells« were observed in suspension cultures of HeLa S3 cells Both cell lines were able to phagocytize latex particles of 5 7 µm mean diameter Nocodazole significantly reduced the formation of »balloon cells« in suspension cultures of JB 1 E cells Transmission electron microscopy indicated that various cytoplasmic membrane components probably could contribute to the membrane material of the vacuoles It is believed that the formation of balloon cells is due to changes in the regulation of the cellular membrane material

Key words Vacuoles suspension culture tumour cells TEM microtubules

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Several observations on cytoplasmic vacuolization in tumour cells leading to the formation of »balloon cells« (i e cells with a single large vacuole and a flattened excentric nucleus) have been published (*Seeger* 1937 *Selby et al* 1955-56 *Wessel & Bernhard* 1957 *Tolnai* 1964 *Burns* 1967 *Chemnitz et al* 1974)

Cytoplasmic vacuolization of ascites tumour cells has commonly been regarded as a sign of cell degeneration a cellular degenerative process forebo ding cell death (*Selby et al* 1955-56 *Wessel & Bernhard* 1957) However mitotic activity of the »balloon cells« and uptake of tritiated thymidine in the nucleus indicate that they are living cells (*Tolnai* 1964 1965 *Chemnitz et al* 1975)

The aim of the present investigation with two different cell lines grown in suspension and monol [illegible]

[illegible]

Our results showed that the JB 1 E cells grown in suspension cultures contained »balloon cells« while they were rarely observed among flattened spindle shaped JB 1 E cells attached to a substrate

MATERIALS AND METHODS

Cells and Growth Media

In the present [illegible] were [illegible] JB 1 E [illegible] tumou[illegible] (B che[illegible] the H[illegible] purchased from GIBCO

Stock cultures of both cell lines were maintained at 37 °C in 75 cm² Nunc flasks in Eagle s minimal essential medium (EMEM-F11) (GIBCO) supplemented with 10% [illegible]

[illegible] (GIBCO) The media were aerated with a mixture of 95% air and 5% CO_2 and changed every other day

Chemicals

Nocodazole (methyl (5 (2 thienylcarbonyl) 1 H

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Light Microscopy

lets of untreated JB 1 E cells cultivated for 24 h in nsion cultures were obtained by centrifugation were fixed in 4% formaldehyde pH 6 8 for ximately 24 h embedded in paraffin and sections lets were stained with one of the following dyes

riodic acid Shiff (PAS) without nuclear staining IcManus and Mowry 1964)
lcian blue (pH 3 and pH 1) (McManus and Mowry 964)

fter 24 h of growth the cultures were fixed using 6 glutaraldehyde buffered with PM buffer (poly zation mix) introduced by *Lufüg et al* (1977) One ption was made concerning some of the spinner s containing 20 × 10^6 cells of the JB 1 E cell line e cells were fixed with 2 5% glutaraldehyde ered with 0 1 M Na-cacodylate buffer 4% sucrose 7 2 (fixative vehicle 300 mOsm) Ruthenium red R) was added to the fixative using the method of t as described by Behnke (1968) The cells were fixed at 37 °C for 2 h and postfixed in 1% OsO_4 for 3 and the further procedure is the same as described for other spinner cultures

Spinner Cultures The cells were fixed at 37 °C for 2– 1 centrifuged for 10 min and the cell pellets were pended in agar dissolved in warm PBS After cooling icewater small cubes of agar were washed in 0 1 odylate buffer (300 mOsm) pH 7 2 and postfixed in 6 OsO_4 in cacodylate buffer pH 7 2 for 1 h at 4 °C ter staining with 1% uranyl in re-distilled water for 1 he cells were dehydrated in increasing conc of acetone d embedded in Araldite

Monolayer Cultures After fixation in 2 5% glutaral hyde in PM buffer for 2–4 h the cells were postfixed % OsO_4 in 0 2 M cacodylate buffer (300 mOsm) pH 2 for 1 h and pre stained with 1% uranyl acetate in re-stilled water for 1 h then dehydrated in series of hanols and embedded in Araldite After polymeriza on selected areas were cut out of the bottom of the unc flasks for sectioning

Ultra thin sections from cultures pre stained with ranyl acetate were contrasted with 0 4% lead citrate at oom temperature for 5 min Ultra thin sections from ultures fixed with RR were contrasted with 1% inc uranyl acetate at 60 °C for 10 min and 0 4% lead itrate at room temp for 5 min

Semi thin sections were cut and stained with 1% olu d ne blue and prepared for light microscopy

RESULTS

Light Microscopy

Spinner and Monolayer Cultures of the JB 1 E Cells Tumour cells with a large vacuole and a flattened excentric nucleus were observed in suspension cultures of the JB 1 E cells (Fig 1a) The percentage of »balloon cells« was approximately 11% (500 cells studied) The vacuoles were not stained by PAS and alcian blue The spindle-shaped cells attached to a solid surface did not contain any large vacuoles (Fig 1b)

Spinner and Monolayer Cultures of Hela S3 Cells Only a few »balloon cells« were observed in suspension cultures of Hela S3 cells (Fig 1c) and none in cells of monolayer cultures (Fig 1d)

Phagocytosis of Latex Particles Approximately 6–8% of the cells of both cell lines were able to phagocytize latex particles of 5 7 µm mean diameter Only few latex particles were found in the large vacuoles in »JB 1 E balloon cells«

JB 1 E Cells Treated with Nocodazole The number of »balloon cells« was significantly reduced in suspension cultures of JB 1 E cells treated with nocodazole Only 1–2% of the cells developed large vacuoles

TEM of »Balloon Cells« in vitro »Balloon cells« formed *in vitro* resembled those observed in the ascites tumour Golgi complex and vesicles were often observed in the vicinity of the vacuole membrane and vesicles often appeared to fuse with the vacuole membrane Microfilaments and microtubules were located around the vacuole microfilaments often radiating out from the vacuole membrane Vesicles and endoplasmic reticulum were found dispersed in the cytoplasm around the vacuole (Fig 2 a–b) The content of the vacuoles was structureless and no remnants of hetero- or autophagocytotic matter were observed The content had a fluid character In specimens stained during the fixation with ruthenium red the cell surface membranes were stained while the membranes of the cellular vacuoles remained unstained (Fig 3)

DISCUSSION

Vacuolization of tumour cells is a general phenomenon and it has been observed in a variety of tumour cells (*Wessel & Bernhard 1957 Tolnai 1964 Burns 1967 Chemnit et al 1974 Bader & Bader* 1976)

Intracellular cysts of breast and gastric carcinoma cells often contain mucous material (*Nevalainen & Järvi* 1976) The Krukenberg tumour consists of metastatic carcinoma cells which are mucous producing The distended cytoplasm and flattened nuclei of individual cells produce a signet ring appearance

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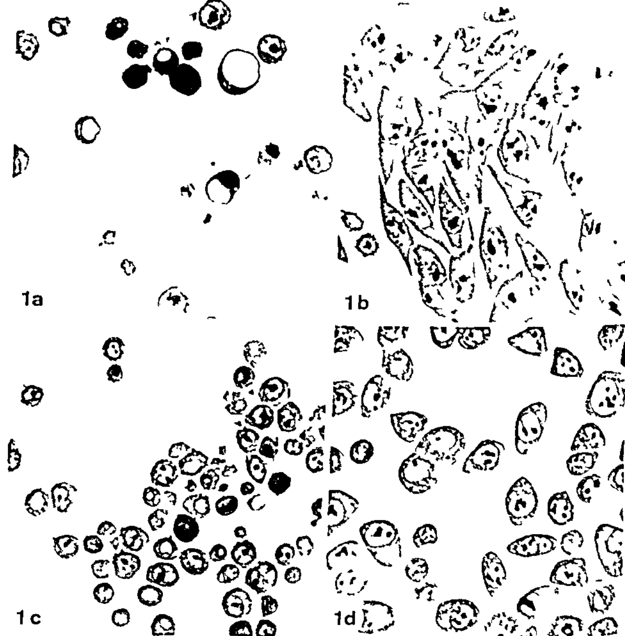


Fig 1a–d a) JB 1 E cells grown in spinner cultures Typ cal balloon cells are present ×400 b) JB 1 E cells grown as a monolayer N balloon cells are seen among the attached sp ndle shaped cells ×400 c + d) Hela S3 cells grown in suspensi n (and as monolayer (d) No balloon cells are present in e ther case ×400

benzimidazol 2 yl) carbona e R 17934) was obtained from Aldrich Chemical Company I C Nocodazole is a new synthetic anticancer drug which interferes with the structure and function of microtubules both in interphase and mitosis (*DeBrabander et al* 1976) Before use nocodazole was dissolved in d methylsulphoxide (DMSO) The usual concentrat on used in the experimen tal stud es was 1 µg/ml culture medium

Experim ental Cultures

After centrifugation 8 × 10^6 cells of the JB 1 E and Hela S3 cell l nes were inoculated separately into Bellco Sp nner flasks contain ng 20 ml culture med um The spinner flasks were aerated and incubated at 37 °C for 24 h stirring speed was set to 200–300 r p m S multaneously with the start of the sp nner cultures cells from the respective stock cultures were inocula ed into Nunc flasks (25 cm) containing 5 ml culture medium The flasks were aerated with a mixture of 5% CO_2 and 95% air and incubated at 37 °C for 24 h Spinner and monolayer cultures had a cell dens ty of 400 000 cells/ml med um To some of the sp nner cultures nocodazole added (1 µg/ml med um) The tumour cells were treated for 24 h

Phagocytos s of Latex Part cles 60 × 10^6 latex particles of 5 7 µm mean d ameter were added to the suspension cultures with 20 × 10^6 JB 1 E or Hela S3 cells

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EM

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[illegible] Araldite After polymerization selected areas were cut out of the bottom of the Nunc flasks for sectioning

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Intracellular cysts of breast and gastric carcinoma cells often contain mucous material (*Nevalainen & Jarvi* 1976) The Krukenberg tumour consists of metastatic carcinoma cells which are mucous-producing The distended cytoplasm and flattened nuclei of individual cells produce a signet ring appearance

Histochemical methods have failed to give a positive reaction for mucous substances of »balloon cells« in ascites tumours (*Tolnai* 1964, *Chemnitz et al* 1974) However, the vacuole membrane was



Fig. 2 a–b a) A section through part of the cytoplasm around the large vacuole (V) in a balloon cell. The Golgi complex (G) and some vesicles are seen together with strands og smooth endoplasmic reticulum. Note the several small vesicles in relation to the Golgi complex and the vacuole membrane. × 6 000. b) A similar section showing that microfilaments are present beneath the vacuole and that some apparently are in association with the vacuole membrane. Microtubules and vesicles (arrowheads) are seen among the filaments. × 78 000

stained with PAS and alcian blue (pH 2.5) indicating the presence of glycoproteins (*Chemnitz et al.* 1974).

The morphology of JB 1 E cells grown in a suspension culture resembles the JB 1 ascites tumour *in vivo* (*Chemnitz et al.* 1974). The balloon cells develop from regular JB 1 E cells indicating that some of the JB 1 E cells are able to develop large vacuoles when grown in suspension contrary to cells attached to a substrate.



Fig 3 This micrograph demonstrates staining of the »balloon cells« with ruthenium red added to the fixative Note the heavy sta ning of the surface membrane of the cell and the complete absence of staining of the membrane of the vacuole (V) ×99 000

The large vacuoles are partly formed by mem brane material accumulated in the cytoplasm Mor phological observations indicate the function of the Golgi complex in this process Other elements of the vacuolar system also seem to contribute

Besides the role of the Golgi complex in the anabolic functioning of the cell the organelle also plays a substantial role in the catabolic functioning of the cell The large vacuoles could be formed by the supplement of membrane material to hetero and autophagocytotic processes in the cytoplasm However preliminary results have failed to show any cytochemical reaction for acid phosphathases in or near the large vacuoles (unpublished observa tions) RR staining indicates that at the time of fixation the large vacuoles are intracellular vacuoles having no communication with the extracellular environment

Phagocytosis of large solid particles (latex beads) does not seem to be coupled with the formation of the large vacuoles

lization could also be a consequence of changes in membrane functions especially in the regulation of membrane transport and/or membrane turnover

Nevertheless our results have shown a conspicu ous difference in tumour cell behaviour due to culture conditions The JB 1 E cell line showed a higher degree of heterogenity than the Hela S3 cell line It is indicated that the cytoplasmic microtubule complex is of importance in the process of vacuole formation although the exact mechanism remains to be elucidated

This study was supported by grants from *Ingemann O Buck s Fund* and *Carla Cornel a Storch Møller s Legat* It is a pleasure to acknowledge the technical assistance of Mrs *E Berg* Mrs *I Holst* Mrs *R Lønstrup* and Mr *E Panton*

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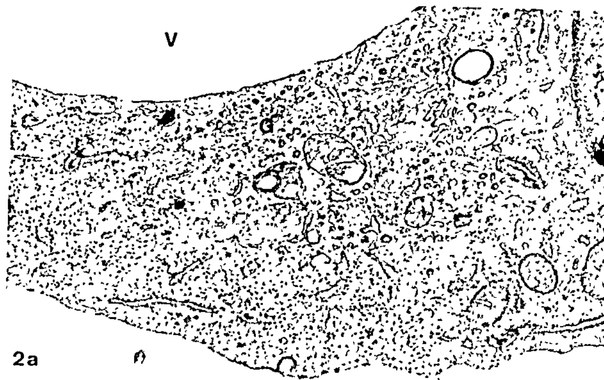
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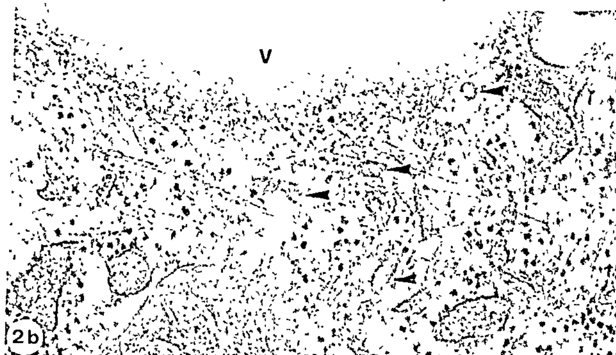


Fig 2 a–b a) A section through part of the cytoplasm around the large vacuole (V) in a »balloon cell« The Golg complex (G) and some vesicles are seen together with strands og smooth endoplasmic reticulum Note the several small vesicles in relation to the Golgi complex and the vacuole membrane × 26,000 b) A similar section showing that microfilaments are present beneath the vacuole and that some apparently are in association with the vacuole membrane Microtubules and vesicles (arrowheads) are seen among the filaments × 78,000

stained with PAS and alcian blue (pH 2 5), indicating the presence of glycoproteins (*Chemnitz et al* 1974)

The morphology of JB-1-E cells grown in a suspension culture resembles the JB-1 ascites tumour *in vivo* (*Chemnitz et al* 1974) »The balloon cells« develop from regular JB-1-E cells, indicating that some of the JB-1-E cells are able to develop large vacuoles when grown in suspension, contrary to cells attached to a s[illegible]

Acta path microbiol scand Sect. A 88 125-127 1980

A SILVER-GOLD IMPREGNATION TECHNIQUE FOR DEMONSTRATION OF STRIATED MUSCLE ELEMENTS

LARS GRIMELIUS and YNGVE OLSSON

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Grimelius L. & Olsson Y A silver gold impregnation technique for demonstration of striated muscle elements Acta path microbiol scand Sect A 88 125-127 1980

A silver gold impregnation technique for demonstration of cross striations in skeletal muscles is presented It is easily performed and all ingredients are chemically well characterized and easily available The method has been successfully applied to formalin fixed paraffin-embedded material from human muscles and can be of value in the histopathological diagnosis of rhabdomyosarcomas and other tumors of muscular origin

Key words Silver gold histochemical technique muscle soft tissue tumors

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For certain diagnostic purposes demonstration of cross-striations in muscles is of crucial importance and optimal staining methods are then required One example is the diagnosis of rhabdomyosarcomas

We have previously described a silver gold impregnation technique for axons and neurofibrillary changes (*Grimelius & Olsson* 1978) and have noted that it could also be used for demonstration of cross-striations in muscles We have now found that the staining is improved by initial oxidation of the sections and would therefore like to present one procedure which in our experience gives optimal results for human muscles

MATERIAL AND METHODS

The cross striations were studied in small samples obtained from autopsies within 24 h after the death of the patient (m m vastus lateralis gastrocnemius biceps brachii In addition we have used mouse diaphragm and gastrocnemius muscle and gained some experience with human soft tissue tumors of muscular origin

The samples measuring about $2 \times 4 \times 8$ mm were fixed in 10% aqueous formalin for 24-78 h at room temperature After dehydration clearing in xylene and embedding in paraffin 5 µm thick longitudinal sections were cut They were treated for 1 min in 0 025% potassium permanganate solved in re-distilled water The sections were then bleached in 20% sodium bisulphite in re distilled water for 15 sec rinsed in 3 baths of re distilled water each for 3 min and finally impregnated in silver gold according to *Grimelius & Olsson* (1978) Control sections were stained without preoxidization All sections were mounted in Canada balsam

RESULTS

In human muscles cross striations could easily be visualized with the original method but oxidation in potassium permanganate followed by bleaching resulted in the most distinct pictures (Fig 1) This variation is therefore our method of choice and can also be applied to mouse muscles and biopsies from human rhabdomyosarcomas (Fig 2)

Under low magnification the cross-striations in skeletal muscles appear as alternating dark and light bands Higher magnification reveals that silver staining is most intense in the periphery of the A bands and in the Z line

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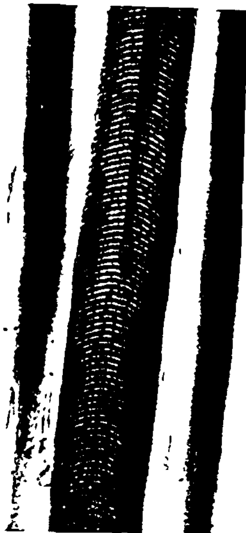


Fig 1 Longitudinal section from human skeletal muscle Cross striations are distinctly visualized Tissue treated according to our proposed method

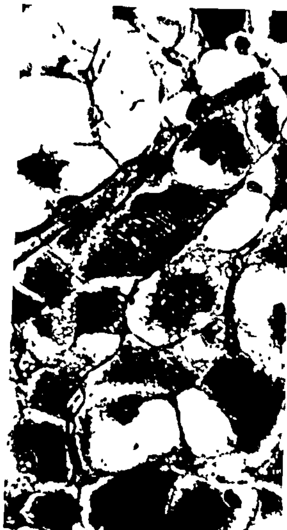


Fig 2 Tissue from a case of rhabdomyoma of adult type The diagnosis of this rare tumor relies *inter alia* on the demonstration of cross striations in tumor cells which in this particular tumor type may be difficult to observe Silver-gold staining according to our proposed method reveals easily visible striations in the cytoplasm of a tumor cell (arrow)

DISCUSSION

The most commonly used staining methods for demonstration of cross striations in skeletal muscle employ iron hematoxylin or phosphotungstic acid hematoxylin (*Adams* 1975) On the basis of our own data and on some other observations in the literature we would suggest metallic impregnation with silver-gold as an alternative when demonstration of cross-striations is required for diagnostic purposes

There are only a few earlier reports on silver methods for visualization of cross-striations in muscle *Dempsey et al* (1946) in a study on the histochemical properties of mammalian striated muscle noted that the Z disc is silver positive when using an ammonical silver nitrate method and the entire isotropic segment was stained by the Bodian procedure *Van de Velde and Brown* (1970) also described a modified version of the Bodian protarg[ol] method for the identification of striated muscl[e] elements

The method described by *Bodian* (1936) relies o[n] silver proteinate (protargol) a chemical substanc[e] not fully chemically characterized The quality o[f] silver proteinate varies therefore often from on[e] batch to another As was pointed out by *Van d[e] Velde and Brown* (1970) the type of protargo[l] determines whether or not staining of muscle wi[ll] be successful For routine use it must therefore be o[f] value to apply a method like ours in which easily obtained well characterized chemicals are used

Acta path microbiol scand Sect A *88* 129–134 1980

MICROFILAMENTS IN CELLS ASSOCIATED WITH INDUCED HETEROTOPIC BONE FORMATION IN GUINEA PIGS

An Immunofluorescence and Ultrastructural Study

RUNE NILSEN

Department of Oral Pathology and Forensic Odontology School of Dentistry University of Bergen 5016 Haukeland Hospital Norway

Nilsen R Microfilaments in Cells associated with induced heterotopic bone formation in guinea pigs An immunofluorescence and ultrastructural study Acta path microbiol scand Sect A *88* 129–134 1980

Sera from patients with active chronic hepatitis were used to demonstrate actin microfilaments in cells of a bone induced model Demineralized and freeze dried allogenic dentine was implanted in the abdominal muscles of Guinea pigs After 21 and 28 days the tissue was excised and showed a) resorption areas of the dentine contained dentinoclasts and macrophages b) osteoid and bone and c) areas of fibroblastic proliferation Osteoblasts and young osteocytes showed the strongest fluorescent staining Electron microscopy of these cells demonstrated abundant 5–7 *nm microfilaments* interpreted as actin in the outer parts and cell projections in which some microtubules also were observed Fewer microfilaments were present in mature osteocytes although the cell projections in mineralized bone also showed microfilaments Microfilaments were also found *in some* dentinoclasts where the actin was located in projections from the ruffled border *protruding into the dentine* The localization in the ruffled border corresponded to the distribution of the fluorescent staining of actin The high amount of actin in osteoblasts and osteocytes may be *correlated to the* synthesis and transport of the new matrix components The microfilaments in the clear sealing zone of dentinoclasts were considered to have a function in the sealing of the ruffled border

Key words Actin bone immunofluorescence transmission electron microscopy

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Microfilaments with a diameter of 5–7 nm are found beneath the plasma membrane of many non muscle cells and it has been shown ultrastructurally that these microfilaments interact with heavy meromyosin (*Ushikawa et al* 1969 *Wessells et al* 1971 *Pollard & Weihing* 1974) The actin-containing microfilaments are suggested to be [illegible] cell mo[illegible] stream[illegible] (*Schroe*[illegible] demonstrated binding of heavy meromyosin to microfilaments in osteoblasts osteocytes and osteoclasts

Smooth muscle antibodies (SMA) appear in serum from [illegible]

[illegible] that this SMA is directed against actin This feature has been used to demonstrate actin by indirect immunofluorescence in smooth muscle (*Johnson et al* 1965) and other cell types (*Groschel Stewart & Groschel* 1974, *Lazarides* 1975)

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Smooth *muscle antibodies* (SMA) appear in serum from patients with *active chronic* hepatitis (*Johnson* [illegible]
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& Ryan [illegible]
shown that this SMA is directed against actin This feature has been used to demonstrate actin by indirect immunofluorescence in *smooth* muscle (*Johnson et al* 1965) and other cell types (*Groschel Stewart & Groschel* 1974 *Lazarides* 1975)

Induction of heterotopic bone formation has been shown to occur after implantation of demineralized allogenic dentine in the abdominal muscles of rats and guinea pigs (*Urist et al* 1967, *Bang* 1973) Previous electron microscopic investigations using this experimental model demonstrated microfilaments peripherally in osteoblasts (*Nilsen* 1977)

In this study the presence and distribution of microfilaments have been studied ultrastructurally and correlated with the actin distribution as demonstrated by indirect immunofluorescence

MATERIALS AND METHODS

Ten young male guinea pigs randomly bred were used Each animal received 4 implants of allogenic demineralized and freeze dried dentine in the pouches of the abdominal muscles (*Bang* 1973) The animals were fed a standard diet (Norwegian standard for guinea pigs and rabbits Statens Institutt for Folkehelse Oslo Norway) supplemented with swedes and hay

After 21 and 28 days respectively the animals 5 in each group were anesthetized with Nembutal[R] and the implants were excised with some surrounding tissue

Electron Microscopy

One part of each implant was divided into small pieces (1 mm^3) (altogether 80 specimens) and subsequently immersed in 2% glutaraldehyde in 0 1 M cacodylate buffer (pH 7 2) containing 7 5% sucrose (some at room temperature and some at + 4 °C) and then postfixed in 2% OsO_4 in the same buffer The specimens were then dehydrated and embedded in Epon 812 One µm thick sections were cut and stained with toluidine blue and the actual cellular zones were chosen prepared and cut at 50–60 nm with a diamond knife on a Reichert Om U 3 ultramicrotome The sections were collected on uncoated grids and double stained with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope

Immunofluorescence

The other part of each implant was directly frozen in isopentane precooled to −140 °C in liqued nitrogen and stored at −80 °C Serum was taken from a patient with chronic active hepatitis containing specific antibodies against muscles (SMA serum) The titre was 1/128 The serum was also tested for antimitochondrial and anti DNA antibodies and showed negative reactions In order to test the reproducibility of the results serum with smooth muscle antibodies from three other patients with chronic active hepatitis was used on all specimens in the same manner Cryostat sections (7 µm) were obtained and washed twice with phosphate buffered saline (PBS) before immersion in cold acetone at + 4 °C for 5 minutes The sections were incubated with SMA serum diluted 1 8 to 1 20 in PBS and incubated in a moist chamber for 60 minutes The preparations were then washed 3 times in PBS for 30 minutes and incubated for another hour in a 1 20 dilution of rabbit antihuman Ig coupled to fluorescein isothiocyanate (FITC) (DAK[illegible] immunoglobulin C[illegible] ratio 2 3 antibody [illegible] then washed 3 ti[illegible] immediately by incident light fluorescence microscop[illegible] (Leitz Ploem opaque illuminator filters 3 5 mm BG [illegible] and K 510)

Control sections were treated with 1 PBS or norm[illegible] human serum 2 SMA serum neutralized by absorptio[illegible] with homogenates of smooth muscle from rat stomach

RESULTS

Transmission Electron Microscopy

The light and electron microscopical study demonstrated three main types of cellular reactions appearing consistently in all animals a) resorption zones with dentinoclasts (multinucleated dentine resorbing giant cells morphologically identical to osteoclasts) and macrophages b) matrix production zones with osteoblasts osteocytes and a few chondroblasts and chondrocytes c) areas consisting of fibroblasts All three zones wer found after 21 days as well as 28 days At 28 days however more mineralized bone was observed For a detailed description of these cells see *Nilsen* (1977) Large amounts of new matrix were found to be produced in all animals

A young connective tissue which surrounded the three zones exhibited only scattered granulocytes and lymphocytes Skeletal muscle fibres occupied the outer parts of the specimens representing the abdominal muscles

Osteoblasts and osteoid osteocytes regularly showed a distinct accumulation of microfilaments along and parallell to the cell membrane facing the newly formed osteoid (Fig 1) These microfilaments had a diameter of 5–7 nm and seemed to be attached to the cell membrane and extended also into cell projections (Fig 2) Quite a few of the cell projections of adjacent cells showed tight junctions Scattered microtubules were observed in both cell projections and the cortical part of the osteoblasts and osteocytes when the specimens were fixed at room temperature Tissue fixed at + 4 °C showed fewer microtubules

Both osteoid and mineralized bone showed abundant microfilaments in the cell projections These microfilaments were longitudinally orientated The mature osteocytes however lacked the distinct accumulation of microfilaments just beneath the cell membrane which was evident in osteoblasts and young osteocytes Deeper parts of the cytoplasm of young matrix producing cells showed smaller bundles of microfilaments often



Fig 1 Peripheral microfilaments in an osteoid osteocyte Microtubule (Arrow) Collagen (C) × 50 000

Fig 2 Longitudinal section fo an extension from an osteoid osteocyte containing parallel microfilaments × 80 000

arranged parallel to the endoplasmatic reticulum

The distribution of microfilaments was less evident in the resorbing cells than in the matrix producing cells Areas of the ruffled border in some of the dentinoclasts however contained bundles of microfilaments These microfilaments were found especially in cellular extensions from the clear sealing zone of the ruffled border (Fig 3) Microtubules however were not found in this area Accumulation of microfilaments was also found in some macrophages Fewer microfilaments were found in undifferentiated cells In a few mature fibroblasts however a subcortical accumulation of microfilaments was demonstrated

Immunofluorescence

In the young undifferentiated connective tissue surrounding the implants a bright green fluorescence was only found in vessels walls A few fibroblasts however exhibited a moderate staining and the skeletal muscles had a strong positive immunofluorescence in the I bands

All the osteoblasts and osteoid osteocytes showed strong cytoplasmic staining for actin (Fig 4) The staining appeared mainly in the periphery of the osteoid osteocytes The osteoblast region exhibited also strong cytoplasmic fluorescence This staining was more diffusely outlined than in osteoid osteocytes Osteocytes in the mineralized bone had just a weak fluorescent staining

In some dentinoclasts a strong intracellular fluorescence was observed in the ruffled border area (Fig 4) Other dentinoclasts however had no evident fluorescence Some macrophages in areas of resorption also showed cytoplasmic fluorescence The cytoplasmic fluorescence staining in the matrix producing cells as well as the positive staining in dentinoclasts was diffusely outlined

All the control preparations were negative and gave only low background fluorescence

DISCUSSION

The cellular reactions to the implanted allogenic dentine including both osteoid and bone formation as well as resorption of dentine confirmed the previous reports of a specific induction of bone (*Urist et al* 1967 *Bang & Urist* 1967 *Bang* 1973 *Nilsen* 1977) The close relationship between the





Fig 3 Microfilaments (arrow) in a cell extensio ruffled border region of a dentinoclast × 36 90

Fig 4 Indirect immunofluorescence showing an staining produced by SMA positive serum N peripheral localization in osteoid osteocytes (a Strong fluorescence was seen in the ruffled border of a dentinoclast (open arrow) lying in a res lacuna in the dentine (D) × 400

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different reaction zones permitted compariso tween the reaction zones within each section

Large amounts of actin were found i periphery of the osteoblasts and young osteocy well as in cell projections of these cells Sn muscle antibodies have not previously been us show actin in bone cells Actin containing micr ments however have previously been demor ted in cultured bone cells with heavy meromyo a specific ultrastructural marker (*King & H* 1975) They suggested that the actin played a r the intracellular transport of small molecules

Microtubules were also found in the a containing areas in matrix producing cells Mic laments and microtubules are proposed to const the contractile part of the cytoskeleton (*Wess al* 1971)

The present study showed the greatest amo of actin in young osteocytes which are a matrix producing cells with only a low mot The accumulation in these cells may then indica function of the microfilaments in the synthesis transport of collagen precursors or other ma components Actin microfilaments in myofil blasts in granulation tissue were recently propo to be involved in synthesis and transport of type collagen (*Gabbiani et al* 1976) Microfilaments recently (*Drenckhahn et al* 1977) suggested to involved in transport and exocytosis in acinar c of salivary glands

The microfilaments and microtubules were c served in the cellular processes which also show tight junctions to other cellular processes fro osteocytes These findings may indicate a possib function of the microfilaments in the transpo between the osteocytes Other electronmicroscopic studies have proposed a possible transport functi of the microfilaments (*Stanka* 1975) and microt bules (*Furseth* 1973) in the cell extensions

The localization and amounts of actin in your osteoid osteocytes in the present report suggest contribution to movements of the osteocytes withi the lacunae These amoeboid movements may hav a role in movements of the extracellular fluid

transport function on account of amoeboid movements of the osteocytes has been proposed previously by *Bassett* (1967)

The microfilaments may then be involved in both extracellular and intracellular transport of new matrix components

The ruffled border of some dentinoclasts in the present study showed a strong fluorescent reaction and ultrastructurally microfilaments were found to protrude into cell projections of the clear sealing zone The observed arrangement of microfilaments in the ruffled border of dentinoclasts corresponded with ultrastructural findings in osteoclasts as reported by *King & Holtrop* (1975) In keeping with *King & Holtrop* (1975) microtubules were not observed in the ruffled border region The ruffled border of dentinoclasts and osteoclasts is highly motile and involved in active phagocytosis (*Gothlin & Ericsson* 1976) The actin distribution observed in the dentinoclasts of the present study could reflect the mobility of these cells

The ruffled border with its clear zone is the active site of resorption of mineralized tissues The clear sealing zone may have a function in making closed environments around the ruffled border in the active resorbing giant cells This would make a reduction of pH possible which would explain the crystal-dissolving function of these giant cells (*Schenk* 1974)

The present study shows that the microfilaments could constitute a contractile part of this sealing area of the ruffled border

The actin microfilaments seem to be involved in a variety of biological processes and this study *suggests* that they are involved in matrix production of bone as well as resorption of hard tissues

I thank Mrs *Turid Davidsen* for expert technical assistance I am also greatly indebted to dr philos *Åge Haugen* for valuable advice and discussion

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STEREOMICROSCOPIC AND HISTOLOGIC CHANGES IN THE COLON OF GUINEA PIGS FED DEGRADED CARRAGEENAN

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Olsen Skov P & Poulsen Seier S Stereomicroscopic and histologic changes in the colon of guinea pigs fed degraded carrageenan Acta path microbiol scand Sect A 88 135-141 1980

A colitis like state was induced in Guinea Pigs fed degraded carrageenan orally By means of a combined semimacroscopic and histologic technique the course of the disease was followed during 28 days The changes were primarily seen and became most prominent in the caecum The first lesions were observed following 24 hours of treatment as small rounded foci initially with degenerative changes and inflammation in the surface epithelium later forming superficial focal ulcerations Ulcerative changes gradually progressed during the experiment forming linear and later large geographical ulcerations Topographically the ulcerative process was strongly related to the larger submucosal vessels Nonulcerated parts of the mucosa were not changed until following 7-14 days of treatment The mucosa became bulging granulated and finally villus like Accumulation of macrophages was found under the surface epithelium after 7-14 days Possible pathogenetic mechanisms are discussed especially the development of the early lesions and the significance of the macrophages

Key words Colon carrageenan stereomicroscopy histology

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Carrageenan is a sulphated polysaccharide derived from the red seaweed Euchema Spinosum It is widely used in the food industry as a stabilizer and *emulgator* (5)

In 1969 *Marcus and Watt* (11 20) reported that a colitis like disease can be induced in laboratory animals by oral administration of degraded carrageenan in the drinking water Undegraded (native) carrageenan was found to have the same effect but was less effective than the degraded form (20)

The colitis inducing effect of degraded carrageenan has been confirmed by other authors (3 8 12 16 19) Guinea pigs and rabbits are the most susceptible species (11 22 23) but colonic changes have been provoked also in rats and mice (11 23) and ... (3)

Several reports (2 3 8 12 19 20 22) have dealt with the histopathology of the colonic lesions induced by degraded carrageenan but the results have been varying and in some cases conflicting both as regards the development of the lesions and their localization *Watt and Marcus* (22) found the changes most pronounced in the caecum but also severe in the rectum and moderate in the rest of the colon *Sharratt et al* (16 17) found the disease almost confined to the caecum whereas *van der Waaij et al* (19) observed the most severe changes in the distal colon In the same way some reports (2 8 16) have considered the well-documented macrophage cytotoxic effect of carrageenan (4) to be the most important factor in the pathogenesis of the ulcerations while other reports (12 19) hardly mention the macrophages

The histopathological technique of investigating gastrointestinal specimens is improved and facilitated by the application of surface staining (9, 13 14 15) Following surface staining the structure of the mucosal surface is visualized *in the stereomicroscope* Changed areas can be localized and subsequently examined histologically

The aim of the present study has been by means of the combined stereomicroscopic and histologic technique to investigate the development of the colonic changes induced by degraded carrageenan and to describe the early lesions and the localization of the disease related to the duration of the treatment

MATERIAL AND METHODS

42 smooth haired Guinea pigs average weight 560 g were given degraded carrageenan 5% in the drinking water They were sacrified in groups of six after 1 2 4 7 14 21 and 28 days During the experiment the animals received Purina Lab Chow *ad libitum* The control material consisted of six untreated Guinea pigs

For preparation of the colonic specimens the Guinea pigs were anaesthetized with Nembutal 50g/100g i p The abdomen was opened and after ligation at the distal end of ileum and rectum the entire colon was fixed *in situ* by direct intraluminal injection of 10% formalin The colon was then removed and placed in formalin After fixation for 10 minutes it was cut open antimesenterically and suspended on a polyethylene plate with fine needles After further fixation for 24 hours the specimens were washed in water and stained with Alcian green 3BX 0 5% in 1% acetic acid for five minutes (9)

The entire specimens were studied under the stereomicroscope Appropriate areas were photographed and subsequently taken out for histological examination The section lines were indicated on stereomicroscopic photographs to permit an exact correlation between the stereomicroscopic and the histologic findings The histological specimens were stained with PAS Hematoxylin Aurentia and also with Alcian blue 1% in 0 1n HCl (pH 1) for identification of macrophages containing carrageenan (7)

RESULTS

In the surface stained specimens the crypt openings and the goblet cells clearly stand out dark green on a bright green background In the controls the pattern of crypt openings was regular (Fig 1) The goblet cells were mainly concentrated in and just around the crypt openings and were only occasionally seen in the surface epithelium The mucosal surface was even

Early Lesions

In the caecum and ascending colon delicate changes were observed already after 24 hours of treatment In the stereomicroscope they were seen as small rounded dark foci 0 5–1 mm in diameter (Fig 2a) The luminal parts of the crypt tubules were seen in the areas as white rings while the interjacent surface was stained dark green by the Alcian In the caecum the small foci were regularly arranged in longitudinal rows closely related to the course of the larger submucosal vessels overlying the three tenia coli In the ascending colon they were observed along the attachment of the mesentary

Histological examination of the small foci (Fig 2b) primarily showed a pronounced flattening of the surface epithelium with disorganization of the epithelial cells lack of nuclear polarity and infiltration with a few leucocytes In areas with changes a little more advanced the crypt epithelium was also affected The crypt tubules seemed to degenerate being narrow and shortend leaving a gap between the base of the crypt and the muscularis mucosae In some areas the crypt tubules had disappeared completely In the lamina propria a moderate infiltration with leucocytes was observed

Ulcerative Changes

After 2 and 4 days epithelial defects were seen in the small foci forming punctuate ulcerations with well-defined edges (Fig 3) The number of foci had increased and they had extended also along the transverse ramifications of the longitudinal vessels (Fig 4)

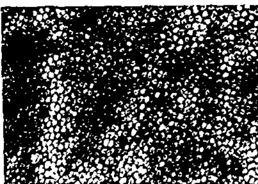
After 7 days many of these small punctuate erosions had become confluent forming shallow linear ulcerations (Fig 4) mainly oriented longitudinally yet following the course of the larger vascular structures

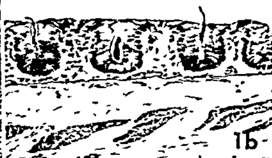
The ulcerative changes gradually progressed and in the fully developed stages as seen after 21 and 28 days of treatment the ulcerations were large irregular and confluent involving large parts of the mucosal surface (Fig 5)

Histologically all the ulcerations were superficial Penetration of the lamina muscularis mucosae was not observed In the bottom of the ulcerations acute inflammatory exudate and granulation tissue was found

The Appearance of the Non Ulcerated Mucosa

Within the first week of treatment the mucosal surface was flat as in the controls After 7 days a local bulging was observed in the mucosa surrounding the ulcerations The remaining surface was normal





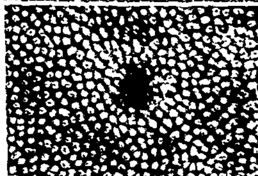








Fig 1a Stereomicroscopic appearance of the normal Guinea pig colon The crypt tubules are seen as white rings with the dark crypt opening in the center (Alcian × 20)

Fig 1b Corresponding histological section showing the appearance of the normal mucosa (PAS-Aurentia × 200)

Fig 2a An early focal lesion observed as a rounded heavily stained area with blurred outlines of crypt openings (Alcian × 20)

Fig 2b Corresponding histological section showing affection of the surface and crypt epithelium The epithelium is infiltrated by inflammatory cells and the epithelial cells are disarranged and flattened (PAS Aurentia × 200)

Fig 3a Punctuate ulceration A well defined superficial lesion surrounded by a mucosa with increased number of goblet cells in the surface epithelium (Alcian × 20)

Fig 3b Corresponding histological section showing superficial ulceration Lamina muscularis mucosa is unaffected (PAS Aurentia × 200)

Diffuse changes in the mucosal surface structure were observed in the caecum and ascending colon after 14 and 21 days They were most pronounced in the caecum where the entire surface was bulging and granulated After 28 days the bulging had progressed giving the surface a villus like appea-

Fig 4 Several punctuate and linear ulcerations following the course of the submucosal vascular structures The mucosa surrounding the ulcerations is granulated (Alcian × 6 3)

Fig 5 Several large ulcerations surrounded by a villus like mucosa (Alcian × 6 3)

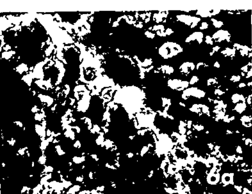




Fig 6a Villus l ke mucosa Crypt openings may be seen between the villus like structures Plenty of goblet cells are seen in the surface epithelium (Alcian × 10)

Fig 6b Correspond ng histological section showing villus like structures Large numbers of PAS-positive macrophages are accumulated in the stroma beneath the surface epithelium (PAS-Aurentia × 200)

rance (Fig 6a) In the ascending colon the changes were less pronounced The surface was swollen slightly granulated often of a gyriform appearance In the distal colon changes were observed only around the ulcerations

Histologically the stroma in non ulcerated areas was normal within the first week In the bulging and villus like mucosa observed in the later stages plenty of large PAS positive macrophages were observed mostly located just beneath the surface epithelium (Fig 6b) The accumulation of macro phages was evident after 7–14 days of treatment Besides there was edema and a moderate infiltra tion with acute and chronic inflammatory cells The space between the crypt tubules was increased but the epithelial cells in the crypt and surface epithelium appeared normal

Localization of Disease

Changes can be classified as focal (ulcerative) and d ffuse (surface contours macrophage infiltration and edema) Focal changes developed considerably earlier than the diffuse changes and in less affected areas they were still the only changes

The caecum was always the most affected part of the colon Small punctuate lesions were observed in the caecum already after 24 hours quickly progres sing to form superficial erosions The ulcerative changes gradually progressed during the 4 weeks of treatment from puncutate to linear and later large confluent ulcerations Diffuse changes in non ulcerated parts of the mucosa were not observed until 14 days after initiation of treatment

In the rest of the colon all changes appeared later and they never became as advanced as in the -- this they had the same morphology Punctuate ulcerations were seen in the proximal colon after 4 days They gradually spread distally and were found in the distal colon and rectum after 21 days In these parts of the colon they remained small never exceeding 2–3 mm in diameter Changes in non ulcerated areas were only moderately developed and were not observed in the distal colon The disease involves the proximal colon in the first place and subsequently spreads distally in continuity to the distal colon

In the rectum however diffuse mucosal inflam mation increased space between the crypt tubules and degeneration of the epithelium might be observed in the later stages

DISCUSSION

When investigating the development of the colonic changes induced by degraded carrageenan it is essential to define the early lesions Applying similar doses of carrageenan the early lesions previously have been described after 5–7 days (2 8 12) A sequence of events preceding ulceration has been proposed (2)

1) Penetration of carrageenan into the lamina propria
2) Accumulation of macrophages containing carra geenan under the surface epithelium
3) Release of lysosomal enzymes from the macro- phages inducing inflammation and edema in the stroma followed by
4) degenerative changes and inflammation in the surface epithelium finally leading to
5) ulceration

In the present investigation early lesions were identified already after 24 hours The first changes observed were

1) Focal affection of the surface epithelium
2) Affection of the crypt epithelium and acute inflammation in the mucosal stroma and finally
3) focal ulceration

Accumulation of macrophages was not observed at this early stage A macrophage reaction was not evident until 7–14 days after initiation of treatment and was mostly observed in the bulging or villus-like mucosa Considering that lesions developed within the first 24 hours of treatment the macrophage accumulation is hardly a part of the primary ulcerative process but rather a secondary foreign body reaction This is moreover supported by the fact that even following direct s c injection of carrageenan accumulation of macrophages is only demonstrated after 5 days (24) Carrageenan is cytotoxic to macrophages (4) It is taken up and stored in the macrophages which are unable to sequester the compound (2 10) Accumulation of macrophages and release of lysosomal enzymes may be involved in the development of the generalized – non ulcerative changes – as the heavy bulging of the mucosa as seen in the fully developed stages This is in agreement with the observation in other studies that the aggregations of macrophages are connected with the bulging and villus like mucosa (2)

It has been discussed how the large carrageenan molecules (MW 30 000) are able to penetrate the intact surface epithelium (2 8) which is nescessary if the macrophage reaction is initiating the ulcerative process The explanation may be that the permeability of the epithelium is increased secondarily to the acute affection of the surface epithelium

This may also be the reason why the focal early changes are detected in the stereomicroscope as areas with increased stainability The large Alcian molecules are probably able to penetrate the damaged epithelium in the focal areas and are retained in the lamina propria staining these areas dark green contrasting to the rest of the mucosal surface where the goblet cells are stained selectively

In the present study strong topographical relationships have been observed between the larger submucosal vascular structures and the initial ulcerative process This relationship has not been noticed in previous studies and we have no explanation for it Further investigation will be needed to evaluate its pathogenetic significans

The reason why the disease is most pronounced in the caecum of the Guinea pig might be that Guinea pigs like other ruminants have a large caecum n which the intestinal contents have a long transit time (18) The mucosal surface in the caecum therefore is exposed earlier and for a longer time the carrageenan than is the rest of the colo Moreover, some of the carrageenan may absorbed or decomposed in the caecum resulting reduced contents of carrageenan in the rest of the colon

It has been well documented that carrageenan is biologically very active compound (6) with sever possible side-effects The important question is on whether it is absorbed or not It has been discusse whether carrageenan might be an etiological facto in human ulcerative colitis (8 11 16 17 21 22 This has been rejected since there are sever pathological differences between experimental an human ulcerative colitis (8 16 17) Moreover onl Guinea pigs and rabbits are sensitive to th undegraded form of carrageenan the large molecules of which do not seem to be absorbed fo instance by rats and monkeys (3 8 10) On th other hand it is possible that carrageenan harmful to patients who already suffer from ulcerative colitis In the present paper it has bee proposed that carrageenan penetrates the surfac epithelium only when this has been damaged primarily In ulcerative colitis atrophy and necrosis are frequently observed in the surface epithelium This may increase the risk of penetration of carrageenan If the widespread application of carrageenan in the food industry should be questioned at all it would be reasonable initially to concentrate on this group of patients in order to find out whether carrageenan is absorbed by them or not and if it is whether it has any harmful effects.

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PROTEOGLYCANS, DNA, AND RNA IN RAT GRANULATION TISSUE, SKIN, AND AORTA BIOCHEMICAL AND HISTOLOGICAL STUDIES

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Proteoglycans DNA and RNA in rat granulation tissue skin and aorta Biochemical and histological studies Acta path microbiol scand Sect A 88 143–150 1980

Proteoglycans in skin and aorta and nucleic acids in skin were studied in rats with subcutaneous sponge implants and in unoperated rats and compared with the sponge induced granulation tissue during a 42 day period In rat aorta there was a predominance of the sulphated glycosaminoglycans in particular heparan sulphate while in skin there was a predominance of hyaluronic acid and dermatan sulphate The subcutaneous sponge implantation caused a decrease in the dry weights of skin and aorta but did not influence the concentrations of the measured variables In aorta the concentrations of chondroitin sulphate and dermatan sulphate decreased with age while in skin there was a slight fall in the concentrations of hyaluronic acid and heparan sulphate with age The rate of biosynthesis of proteoglycans as estimated from the ^{35}S sulphate uptake was higher in skin than in aorta A sequential development was noticed in granulation tissue with an initial high content of hyaluronic acid followed by increasing amounts of chondroitin sulphate and dermatan sulphate in older granulation tissue The RNA/DNA ratio reached a maximum in 14 day old granulation tissue but was unchanged in skin throughout the 42 day period It is concluded that the distribution of proteoglycans and nucleic acids differs from tissue to tissue This may be of relevance for tissue differences in susceptibility to diseases and to the effects – and side-effects – of drugs The morphological and biochemical development of sponge induced granulation tissue from rats was similar to other types of granulation tissue during the first 4 weeks

Key words Proteoglycans nucleic acids granulation tissue rats

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The inflammatory process and the production of granulation tissue is part of the normal biological defence mechanism The production of chronic granulation tissue however may also be one of the pathogenic alterations in diseases such as the rheumatic diseases Information on the composition of connective tissue and granulation tissue may promote the understanding of the connective tissue diseases and be a guidance to appropiate medical treatment In a previous study (*Hansen* 1975) the collagen development in granulation tissue in subcutaneously implanted viscose cellulose sponges was compared with collagen of skin and aorta from rats implanted with sponges and from unoperated rats

The purpose of the present investigation was to compare the proteoglycans and nucleic acids in granulation tissue skin, and aorta of rats during a 42 day period after the induction of granulation

TABLE I *Dry Defatted Weight (DDW) of Aorta (A) Skin Samples (S) and Granulation tissue (G) and the Concentration of Glycosaminoglycans (μg Hexosamine per mg DDW) and of RNA and DNA (μg Ribose and Deoxyribose per mg DDW) and $^{35}SO_4$ Incorporation in Glycosaminoglycans*

			Days after sponge implantation							SD	P_{time}
			0	4	8	14	22	33	42		
DDW	A	−O	12 1	15 0	17 6	21 7	24 0	27 2	28 2	3 7	<0 01
		+O		13 8	16 2	19 1	23 5	24 2	27 5		
	S	−O	185	195	215	217	239	318	339	34	<0 01
		+O		180	170	197	246	302	313		
	G			71	137	127	135	183	181	28	<0 01
HA	A		0 26	0 43	0 21	0 22	0 20	0 26	0 17	0 24	n s
	S		0 44	0 45	0 48	0 45	0 39	0 37	0 40	0 08	<0 05
	G			0 54	0 45	0 40	0 31	0 21	0 24	0 12	<0 01
ChS	A		0 70	0 60	0 62	0 57	0 46	0 45	0 44	0 11	<0 01
	S		0 14	0 12	0 13	0 12	0 13	0 13	0 13	0 02	n s
	G			0 22	0 34	0 63	0 48	0 32	0 35	0 13	<0 01
DS	A		0 66	0 66	0 57	0 62	0 54	0 53	0 45	0 10	<0 01
	S		0 33	0 34	0 33	0 40	0 41	0 35	0 40	0 06	n s
	G			0 05	0 16	0 40	0 48	0 65	0 58	0 14	<0 01
HS	A		0 76	0 78	0 77	0 79	0 89	0 84	0 87	0 19	n s
	S		0 11	0 12	0 09	0 10	0 09	0 08	0 09	0 02	<0 01
	G			0 07	0 08	0 08	0 10	0 12	0 13	0 05	<0 05
RNA	S		3 82	2 99	2 61	1 81	1 86	1 57	1 16	0 26	<0 01
	G			2 86	4 95	6 86	7 10	6 92	7 22	1 34	<0 01
DNA	S		2 15	1 82	1 54	1 01	1 05	0 92	0 63	0 20	<0 01
	G			4 23	3 33	3 56	4 14	4 46	4 65	0 82	<0 01
RNA/DNA	S		1 84	1 65	1 70	1 80	1 81	1 72	1 86	0 21	n s
	G			0 70	1 47	1 92	1 72	1 56	1 56	0 12	<0 01
^{35}S cpm per mg DDW: ChS	A		11 4			5 3			3 8	1 3	<0 01
	S		6 3			4 8			4 8	1 2	<0 01
DS + HS	A		12 6			6 1			4 2	1 4	<0 01
	S		19 3			18 0			15 5	3 8	<0 05
ChS + DS + HS	G					9 1			10 0	2 4	n s

HA hyaluronic acid ChS chondroitin 4/6 sulphate DS dermatan sulphate HS heparan sulphate −/+O −/+ operation with subcutaneous sponge implantation SD standard deviation within the groups P values derived by an analysis of variance n s not statistically significant (P > 0 05) (4–24 animals per group)

0 05 $P_{a \times n}$ < 0 01) There was no detectable effect of the operation on the concentrations or total amounts of glycosaminoglycans or on the $^{35}SO_4$ uptake in aorta *In skin* the concentrations of the glycosaminoglycans the $^{35}SO_4$ uptake and RNA and DNA were unaffected by the sponge implantation whereas the total content per skin sample of hyaluronic acid *dermatan* sulphate and heparan sulphate was decreased in accordance with the decrease in the total dry weight of the skin

tissue Skin and aorta from operated and unoperated rats were compared Furthermore histological studies of the connective tissue development in subcutaneously implanted viscose cellulose sponges in rats were performed Sponge granulation tissue from rats was compared with granulation tissue from rabbit aorta previously described (*Helin et al* 1971)

MATERIALS AND METHODS

108 male Sprague Dawley rats weighing 140–150 g were used At the beginning of the experiment each of 48 rats had 2 viscose cellulose sponges of 1 × 1 × 2 cm implanted subcutaneously symmetrically in the infrascapular region (*Viljanto* 1964) On the same day 12 un operated animals were killed by decapitation On the 4th 8th 22nd and 33rd day after the operation 6 operated and 6 unoperated animals were decapitated On the 14th and 42nd day 12 operated and 12 un-operated animals were decapitated The rats killed at the beginning of the experiment and on the 14th and 42nd day received 48 hours before decapitation 100 μCi ^{35}S sulphate in 1 ml of physiological saline containing 120 μg un labelled sodium sulphate

Biochemical Analyses

The subcutaneously implanted sponges with their contents of granulation tissue were removed and freed from adherent connective tissue Circular pieces of skin of 2 1 cm in diameter were taken from the lumbar regions 1–2 cm away from the skin wound in the sponge bearing rats The hair of the skin was cut with an electrical hair cutter and the subcutaneous fat removed with a scalpel The entire aorta from the aortic valve to the bifurcation was removed and the adventitia stripped off All the samples were freeze dried ground with a Wiley mill defatted with chloroform/methanol (1/3) and dried *in vacuo*

The glycosaminoglycans were isolated and determined as described by *Thunell* (1967) Samples were digested with papain for 6 hours at 67° and centrifuged to remove un digested material Cetylpyridinium chloride was added to the supernatant The precipitate was redisolved in n propanol and again precipitated with ethanol as sodium salts This precipitate was digested with hyaluronidase (testicular Leo Halsingborg activity 15 000–20 000 IU/mg) overnight after which the glycosaminoglycans were fractionated on cellulose columns into depolymerized and non depolymerized fractions In samples from granulation tissue the salt precipitates were digested with nucleases before the hyaluronidase digestion because of the high content of nucleic acids

The hexosamines were liberated by hydrolysis and the content of glucosamine and galactosamine in skin and aorta was determined by the use of a gas chromatograph (90 cm column 0 3 cm diameter SR 2300 100/120 Mesh 205 °C) after transformation of the hexosamines to their alditolacetates dissolved in dimethylformamide From granulation tissue the glucosamine and galactosamine were separated on a Dowex 50 microcolumn (*Antonopoulos* 1966) and determined as hexosamines (*E[illegible] & Morgan* 1933)

The total activity of ^{35}S sulphate in glycosamino[illegible]cans in granulation tissue was determined after pa[illegible] digestion and precipitation of the glycosaminogly[illegible] with cetylpyridinium chloride In skin and aorta the [illegible] sulphate activities were determined separately in dep[illegible]merized and non-depolymerized glycosaminoglyc[illegible] after the hyaluronidase digestion All counts w[illegible] performed in a liquid scintillation spectrometer (Pack[illegible] *model 3385*)

The determinations of DNA and RNA were o[illegible] performed in skin and granulation tissue owing t[illegible] shortage of aortic tissue The samples were boiled for [illegible] minutes to inactivate nucleases before digestion w[illegible] papain After papain digestion the nucleic acids w[illegible] precipitated with cadmium and the content of DNA a[illegible] RNA determined according to the method of *Hatcher [illegible] Goldstein* (1969)

Histological Preparations

A 2 mm thick slice was cut from the middle of one [illegible] the implanted sponges from each animal The tissue w[illegible] fixed in 0 5% cetylpyridinium chloride in 4% formal[illegible] (*Williams & Jackson* 1956) and the specimens we[illegible] orientated in the paraffin so as to allow distincti[illegible] between the superficial and the profound surfaces of t[illegible] granulation tissue in the sponges

The 6–8μ thick sections were stained using t[illegible] following staining methods

1 Haematoxylin and eosin
2 Van Gieson stain for *collagen fibers* (*Lill e* 1965)
3 Orcein staining for *elastic fibers* (*Lillie 1965*)
4 *Acid glycosaminoglycans* were stained with
   a 0 3% Alcian Blue 8GX (*G T Gurr*) at pH 1 an[illegible] pH 2 5 (*Pearse* 1968) in order to distinguish betwee[illegible] weakly and strongly sulphated mucosubstances
   b 0 1% toluidine blue 0 (Merck) in 30% ethano[illegible] (*Kramer & Windrum* 1955)
   c 0 1% toluidine blue in McIlvain buffers at pH [illegible] and pH 3 and in Walpole buffers at pH 2 and pH [illegible] (modified after *Krygier and Kasprzyk 1961*)
5 A combined method for acid glycosaminoglycans elastic fibers collagen fibers and nuclei (*Garbarsch* 1973)
6 The Calcium red method of McGee Russell as described by *Pearse* (1972) for calcium deposits

The ingrowth of tissue into the sponges was measured on the cross sections with a Leitz ASM Image Analysis System at a magnification of 40× The ingrowth was determined as the mean of the maximum ingrowth in 2 dimensions

RESULTS

Biochemical Observations

The biochemical results are given in Table I and Fig 1 and 2 The defatted dry weight of the aorta and skin samples was slightly smaller in sponge bearing rats than in unoperated animals (P_{aorta} <

ABLE I *Dry Defatted Weight (DDW) of Aorta (A) Skin Samples (S), and Granulation tissue (G) and the concentration of Glycosaminoglycans (μg Hexosamine per mg DDW) and of RNA and DNA (μg Ribose and Deoxyribose per mg DDW) and $^{35}SO_4$ Incorporation in Glycosaminoglycans*

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		G		*0 54*	*0 45*	*0 40*	*0 31*	*0 21*	0 24	0 12	<0 01
ChS		A	0 70	0 60	0 62	0 57	0 46	0 45	0 44	0 11	<0 01
		S	0 14	0 12	0 13	0 12	0 13	0 13	0 13	0 02	n s
		G		0 22	0 34	0 63	0 48	0 32	0 35	0 13	<0 01
DS		A	0 66	0 66	0 57	0 62	0 54	0 53	0 45	0 10	<0 01
		S	0 33	0 34	0 33	0 40	0 41	0 35	0 40	0 06	n s
		G		0 05	0 16	0 40	0 48	0 65	0 58	0 14	<0 01
HS		A	0 76	0 78	0 77	0 79	0 89	0 84	0 87	0 19	n s
		S	0 11	0 12	0 09	0 10	0 09	0 08	0 09	0 02	<0 01
		G		0 07	0 08	0 08	0 10	0 12	0 13	0 05	<0 05
RNA		S	3 82	2 99	2 61	1 81	1 86	1 57	1 16	0 26	<0 01
		G		2 86	4 95	6 86	7 10	6 92	7 22	1 34	<0 01
DNA		S	2 15	1 82	1 54	1 01	1 05	0 92	0 63	0 20	<0 01
		G		4 23	3 33	3 56	4 14	4 46	4 65	0 82	<0 01
RNA/DNA		S	1 84	1 65	1 70	1 80	1 81	1 72	1 86	0 21	n s
		G		0 70	1 47	1 92	1 72	1 56	1 56	0 12	<0 01
cpm mg W	ChS	A	11 4			5 3			3 8	1 3	<0 01
		S	6 3			4 8			4 8	1 2	<0 01
	DS + HS	A	12 6			6 1			4 2	1 4	<0 01
		S	19 3			18 0			15 5	3 8	<0 05
	ChS + DS + HS	G				9 1			10 0	2 4	n s

HA hyaluronic acid ChS chondroitin 4/6 sulphate DS dermatan sulphate HS heparan sulphate −/+O −/+ operation with subcutaneous sponge implantation SD standard deviation within the groups P values derived by an analysis of variance n s not statistically significant (P>0 05) (4–24 animals per group)

0 05 P $_{o.n}$ < 0 01) There was no detectable effect of the operation on the concentrations or total amounts of glycosaminoglycans or on the $^{35}SO_4$ uptake in aorta In skin the concentrations of the glycosaminoglycans the $^{35}SO_4$ uptake and RNA and DNA were unaffected by the sponge implantation whereas the total content per skin sample of hyaluronic acid *dermatan* sulphate and heparan sulphate was decreased in accordance with the decrease in the total dry weight of the skin

tissue Skin and aorta from operated and unoperated rats were compared Furthermore, histological studies of the connective tissue development in subcutaneously implanted viscose cellulose sponges in rats were performed Sponge granulation tissue from rats was compared with granulation tissue from rabbit aorta previously described (*Helin et al* 1971)

MATERIALS AND METHODS

108 male Sprague-Dawley rats weighing 140–150 g were used At the beginning of the experiment each of 48 rats had 2 viscose cellulose sponges of 1 × 1 × 2 cm implanted subcutaneously symmetrically in the infrascapular region (*Viljanto* 1964) On the same day 12 unoperated animals were killed by decapitation On the 4th, 8th, 22nd and 33rd day after the operation 6 operated and 6 unoperated animals were decapitated On the 14th and 42nd day 12 operated and 12 un-operated animals were decapitated The rats killed at the beginning of the experiment and on the 14th and 42nd day received 48 hours before decapitation 100 μCi ^{35}S sulphate in 1 ml of physiological saline containing 120 μg un-labelled sodium sulphate

Biochemical Analyses

The subcutaneously implanted sponges with their contents of granulation tissue were removed and freed from adherent connective tissue Circular pieces of skin of 2 1 cm in diameter were taken from the lumbar regions 1–2 cm away from the skin wound in the sponge bearing rats The hair of the skin was cut with an electrical hair cutter and the subcutaneous fat removed with a scalpel The entire aorta from the aortic valve to the bifurcation was removed and the adventitia stripped off All the samples were freeze-dried ground with a Wiley mill defatted with chloroform/methanol (1/3) and dried *in vacuo*

The glycosaminoglycans were isolated and determined as described by *Thunell* (1967) Samples were digested with papain for 6 hours at 67° and centrifuged to remove un digested material Cetylpyridinium chloride was added to the supernatant The precipitate was redisolved in n-propanol and again precipitated with ethanol as sodium salts This precipitate was digested with hyaluronidase (testicular Leo Halsingborg activity 15 000–20 000 IU/mg) overnight after which the glycosaminoglycans were fractionated on cellulose columns into depolymerized and non depolymerized fractions In samples from granulation tissue the salt-precipitates were digested with nucleases before the hyaluronidase digestion, because of the high content of nucleic acids

The hexosamines were liberated by hydrolysis and the content of glucosamine and galactosamine in skin and aorta was determined by the use of a gas chromatograph (90 cm column, 0 3 cm diameter, SR 2300 100/120 Mesh, 205 °C) after transformation of the hexosamines to their alditolacetates dissolved in dimethylformamide From granulation tissue the glucosamine and galactosamine were separated on a Dowex-50 microcolumn (*Antonopoulos* 1966) and determined as hexosamines (*Elson & Morgan* 1933)

The total activity of ^{35}S sulphate in glycosaminoglycans in granulation tissue was determined after papain digestion and precipitation of the glycosaminoglycan with cetylpyridinium chloride In skin and aorta the ^{35}S sulphate activities were determined separately in depolymerized and non depolymerized glycosaminoglycans after the hyaluronidase digestion All counts were performed in a liquid scintillation spectrometer (Packard model 3385)

The determinations of DNA and RNA were only performed in skin and granulation tissue owing to a shortage of aortic tissue The samples were boiled for 15 minutes to inactivate nucleases before digestion with papain After papain digestion the nucleic acids were precipitated with cadmium and the content of DNA and RNA determined according to the method of *Hatcher & Goldstein* (1969)

Histological Preparations

A 2 mm thick slice was cut from the middle of one of the implanted sponges from each animal The tissue was fixed in 0 5% cetylpyridinium chloride in 4% formalin (*Williams & Jackson* 1956) and the specimens were orientated in the paraffin so as to allow distinction between the superficial and the profound surfaces of the granulation tissue in the sponges

The 6–8μ thick sections were stained using the following staining methods

1 Haematoxylin and eosin
2 Van Gieson stain for collagen fibers (*Lillie* 1965)
3 Orcein staining for elastic fibers (*Lillie* 1965)
4 Acid glycosaminoglycans were stained with
   a 0 3% Alcian Blue 8GX (G T Gurr) at pH 1 and pH 2 5 (*Pearse* 1968) in order to distinguish between weakly and strongly sulphated mucosubstances
   b 0 1% toluidine blue 0 (Merck) in 30% ethanol (*Kramer & Windrum* 1955)
   c 0 1% toluidine blue in McIlvain buffers at pH 5 and pH 3, and in Walpole buffers at pH 2 and pH 1 (modified after *Krygier and Kasprzyk* 1961)
5 A combined method for acid glycosaminoglycans elastic fibers collagen fibers and nuclei (*Garbarsch* 1973)
6 The Calcium red method of McGee Russell as described by *Pearse* (1972) for calcium deposits

The ingrowth of tissue into the sponges was measured on the cross sections with a Leitz ASM Image Analysis System at a magnification of 40× The ingrowth was determined as the mean of the maximum ingrowth in 2 dimensions

RESULTS

Biochemical Observations

The biochemical results are given in Table 1 and Fig 1 and 2 The defatted, dry weight of the aorta and skin samples was slightly smaller in sponge-bearing rats than in unoperated animals (P_{aorta} <

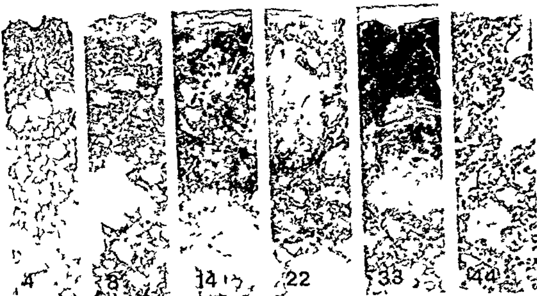


Fig 4 Ingrowth of granulation tissue into subcutaneously implanted viscose cellulose sponges in rats during a 42 day period (Tolu d ne blue × 18) Numbers refer to days after the sponge implantation

that 50% of the total granulation tissue had been produced already 8 days after the sponge implantation and 90% after 22 days

A detailed description of the development of granulation tissue in the viscose cellulose sponge has been given by *Holund et al* (1979)

The sponge consisted of irregular outlined cellulose trabeculas unreactive to the stains employed

Four days after the implantation the interior of the sponge contained PAS positive amorphous material and numerous leucocytes Capillaries and fibroblasts proliferated into the sponge behind a front of macrophages and a vanishing PTAH positive fibrinous network A collagen capsule surrounded the sponge containing dispersed mast cells stained with orcein PTAH Alcian blue at pH 1 and 2 5 and metachromatically with toluidine blue at all pH levels In the capsule and the proliferating zone of fibroblasts spots of amorphous intercellular substance alcianophilic at pH 1 and 2 5 and metachromatic below pH 5 accumulated Some foreign body giant cells were lodged on the peripheral trabeculas

On day 8 the capsule was more prominent with coarser collagen fibers and numerous arterioles Among the proliferating fibroblasts dispersed granulocytes and mononuclear cells were noticed The polymorphous leucocytes in the sponge exhibited [illegible] Foreign body giant [illegible] ibeculas apparently originating as fusing macrophages Areas with metachromasia at pH 7 and pH 5 and alcianophilia were observed between the prolifera



Fig 5 Macrophages in the front of the proliferation of granulation tissue after 14 days of sponge implantation The macrophages conta n dis ntegrated material from the interior of the sponge (Toluid ne blue × 320)

A marked tissue difference was noticed in the glycosaminoglycan pattern (Table 1) In aorta, there was a predominance of the sulphated glycosaminoglycans, in particular heparan sulphate, while the concentration of hyaluronic acid was lower in aorta than in skin and granulation tissue In skin, there was a predominance of hyaluronic acid and dermatan sulphate In granulation tissue, the glycosaminoglycan pattern changed distinctly with age A high concentration of hyaluronic acid was noticed during the first week of granulation tissue production and fell thereafter The concentration of chondroitin 4/6 sulphate reached a maximum at 2 weeks while the concentration of dermatan sulphate reached a maximum after 4 weeks The concentration of heparan sulphate was small in granulation tissue in comparison with the other glycosaminoglycans The total contents of the glycosaminoglycans in granulation tissue showed the same maxima as the concentrations (Fig 1) In aorta there was a fall in the concentrations of chondroitin 4/6 sulphate and dermatan sulphate, while in skin there was a fall in the concentrations of hyaluronic acid and heparan sulphate with time The total contents of the different glycosaminoglycans, however increased with time in both aorta and skin

The incorporation of ^{35}S sulphate in the glycosaminoglycans per mg dry defatted weight decreased with time in aorta and skin while there was no difference in granulation tissue between 14 and 42 days The incorporation of ^{35}S sulphate was higher in dermatan sulphate + heparan sulphate than in chondroitin 4/6 sulphate in skin but not in aorta The total incorporation of $^{35}SO_4$ per mg dry defatted weight was higher in skin than in aorta and

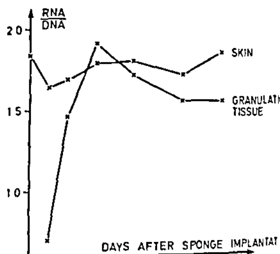


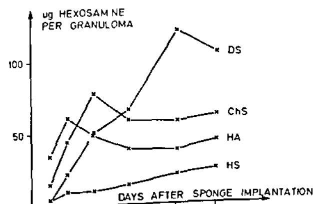
Fig 2 RNA/DNA ratio in skin and sponge induc granulation tissue in rats

granulation tissue The total $^{35}SO_4$ uptake in t glycosaminoglycans per sample decreased in aor with time but increased in skin and granulati tissue

The concentrations and total amounts of DN and RNA decreased with time in skin and increas in granulation tissue There was no change wi time in RNA/DNA ratio in the skin while granulation tissue the ratio increased to a maximu at 14 days (Fig 2)

Histological Observations

The one-dimensional ingrowth of granulati tissue into the sponge reached a maximum after days of implantation (Fig 3 and 4) A calculation the three dimensional ingrowth however show



...osam noglycans in sponge ...onge ...froi implantation ... tin sulphate 4 and 6 DS = dermatan sulphate HS = heparan sulphate

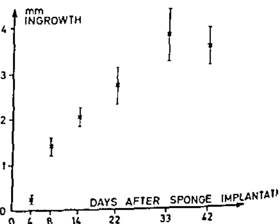


Fig 3 Ingrowth of granulation tissue into subcutane usly implanted viscose cellulose sponges in rats during 42 day period The bars indicate the standard deviati of the means

The morphological development of granulation tissue in viscose cellulose sponges in the present investigation accords with that of previous investigations (*Thomasson et al* 1973, *Pallin et al* 1975) but in these studies giant cells are not mentioned This lack of giants cells may be due to differences in sizes of pores (*Salvatore et al* 1961) or other physical characteristics of the sponges (*Bing* 1955) since the sponge material itself was the same The histology of the granulation tissue in viscose sponge and in different plastic sponge materials (*Bing* 1955 *Edwards et al* 1957 *Kelly* 1962 *Bole et al* 1962 *Wiener et al* 1975) also corresponds mutually although there are a few differences (*Hølund et al* 1979)

The glycosaminoglycans in the sponge are histochemically reflected by the metachromasia and the alcianophilia (*Bole et al* 1962 *Kelly* 1962) In the present investigation however the metachromasia did not correspond well with the content of sulphated glycosaminoglycans in the intercellular substance in contradistinction to the alcianophilia This may be due to the competitive protein effect on the anions at low pH levels (*Kelly* 1955 *Andersen and Ehlers* 1967) and to binding of the sulphated glycosaminoglycans to the collagen (*Sugiyama* 1972)

In conclusion the content and distribution of glycosaminoglycans and nucleic acids differs from tissue to tissue The sequential development of glycosaminoglycans and the histological observations are similar in granulation tissue from different sources although the duration of the production of granulation tissue may depend on the tissue and the type of induction of inflammation

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Fig 6 Fibrinoid substance in 33 day old granulation tissue (PTAH × 100)

ting centrally located cells besides a general weak alcianophilia at pH 1 and pH 2 5

On day 14 peripheral giant cells filled with pyknotic nuclei seemed to disintegrate in some places and mononuclear cells accumulated around the trabeculas in these areas The macrophages at the front of the proliferating fibroblasts contained nuclear material from the leucocytes in the interior of the sponge (Fig 5)

On day 22 lymphocytes and plasma cells were observed the fibroblasts had propagated further centrally and the number of leucocytes in the sponge interior was diminished Accumulation of alciano philic amorphous intercellular sucstance was ob served, but no metachromasia was seen below pH 2

On day 33 and 42 most of the sponges were filled with connective tissue and the central accumulation of leucocytes vanished Fat cells extended from the capsule into the outer part of the loose connective tissue in the sponge In some sponges »fibrinoid« substances characterized by eosinophilia PTAH-positivity and a weak PAS-positivity were observed (Fig 6)

DISCUSSION

As for collagen (*Bailey & Robins* 1973) differences in glycosaminoglycan pattern between tissues are greater than the differences between the same tissues from different species (*Stefanovich & Akiyama* 1970, *Uitto et al* 1971, *Helin et al* 1971)

The relatively higher rate of synthesis of dermatan sulphate and heparan sulphate in skin than in aorta as estimated from the $^{35}SO_4$ incorporations of these proteoglycans is consistent with a hi turnover in skin than in aorta The decrease time in the concentrations of hyaluronic acid heparan sulphate in skin and of chondroitin sulphate and dermatan sulphate in aorta is in due to a dilution effect caused by increa amounts of for example, collagen and ela (*Hansen* 1975) as the total content of glycosam glycans in skin and aorta increased with the ti Although the cell content per skin area as estim from the DNA and RNA content decreased v time, the ability of proteoglycan synthesis (^{35} uptake) was increased This indicates either that decrease in cell content is confined to other cell ty than those synthesizing proteoglycans or that capacity of the individual cells to synthes proteoglycans increases with time

In granulation tissue the initial high level DNA and low RNA/DNA ratio may be explai by a high content of leucocytes which do synthesize protein This is in accordance w experiments by *Pallin et al* (1975) who aspiral leucocytes from sponge granulation tissue a determined the DNA and RNA content T maximum RNA/DNA ratio in granulation tiss was achieved after 14 days at the time of maximu collagen synthesis (*Lampiaho & Kulonen* 1967)

The variation with time of glycosaminoglycans granulation tissue (Fig 1) is in agreement wi other studies of granulation tissue (*Berenson an Dalferes* 1960, *White et al* 1961, *Kirckheimer* 196 *Bentley* 1968 *Reid & Flint* 1974), but differs fro the observations by *Lehtonen* (1968) This differen may be explained by interference from nucleic aci (*Thunell* 1967) which in the present study wa overcome by digestion with nucleases

The production of granulation tissue in th subcutaneously implanted viscose cellulose sponge seemed to cease after 33 days (Fig 1 3 and 4) Thi is in contrast to the injured rabbit aorta where th production of granulation tissue and proteoglycan continued beyond 60 days (*Helin et al* 1971) Th difference may be due either to the continuou haemodynamic stimulus of the vascular wall or to a different reactivity of the smooth muscle cells in a bradytrophe tissue The production of proteoglycans with time in sponge granulation tissue was similar to that of granulation tissue of aorta Morphological studies also showed the same phases in the inflammatory reaction and fibroproductive proces ses (*Helin et al* 1971, *Garbarsch* 1973)

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PULMONARY BLASTOMA

A Clinico-Pathological Study of Eleven Cases

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Eleven cases of pulmonary blastoma are presented the largest series reported from one department to date The frequency of pulmonary blastoma was found to be 0 5 per cent of all primary lung cancers The variegated histologic appearance of the tumour and the diverging metastatic pattern are described in detail In light of the complex histological picture and the biological behaviour a suggestion of the histogenesis of pulmonary blastoma is attempted

Key words Pulmonary blastoma frequency

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Pulmonary Blastoma (PB) is a very rare primary lung tumour consisting of a mixture of immature epithelial and mesenchymal components The tumour was first reported in 1945 by *Barrett and Barnard* (2) and was again reported by *Barnard* in 1952 (1) In 1961 *Spencer* (25) added three further cases and defined the tumour entity He drew attention to the similarities to nephroblastoma and suggested the commonly used term pulmonary blastoma Indeed based on the number of cases previously reported it would seem to be rare however between 1971 and 1978 11 cases were diagnosed from amongst the lung tumour cases at the Department of Pathology at Bispebjerg Hospital In view of this unprecedented number of cases we are prompted to describe their clinico pathological findings

MATERIAL AND METHODS

During the period 1971–1978 nine cases of PB were diagnosed at Bispebjerg Hospital and another two typical cases were revealed by a survey of the microscopic material from all malignant primary lung tumours diagnosed during the same period as sarcomas or mixed tumours The criterion for the diagnosis PB was a tumour consisting of both an immature mesenchymal and an epithelial component in accordance with the description given by *Spencer* (26) Monotypic or doubtful cases of PB were not included in this material For comparison all malignant primary lung tumours diagnosed at Bispebjerg Hospital from 1973 to 1975 were registered and recorded as to histological type (17)

RESULTS

In Table 1 the 11 cases are presented Two were women and nine men and the mean age was 67 years

Ten patients had a brief duration of symptoms One patient (case 2) had had a known atelectasis for seven years before severe expectoration led to resection despite an impaired lung function In one patient (case 8) a lung infiltrate was found [illegible] out to both lungs confirmed at autopsy two weeks later In two patients (cases 10

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and 11 lung infiltrates were diagnosed during investigation of arteriosclerotic heart disease which made them inoperable

The tumours were right sided in seven cases and left-sided in four In eight cases the tumour was confined to one lobe in one case to two neighbouring lobes and in two cases the tumour involved parts of all three lobes of the right lung

Gross Pathology

The tumours were rounded rather well demarcated and surrounded by a pseudocapsule of compressed lung tissue or bronchial wall which further investigation showed to be invaded by tumour growth The tumour size ranged from 2 5 cm to 10 cm in diameter In five cases (cases 2 3 5 8 9) the tumours showed partly intrabronchial growth part of which appeared as smooth polypoid grape like processes free of the bronchial mucosa In one case (case 10) a similar smooth finger like process was seen in a branch of a pulmonary vein extending into the left auricle of the heart The cut surface of the tumours was smooth and bulging and on inspection was greyish white to pink with scattered haemorrhages and necroses The concistency was rather soft In two cases (cases 2 and 4) a central part of the tumour matched the above description the surrounding tumour tissue being greyish to yellow and rather dry with a plane cut surface

Microscopic Findings

Some histologic features of the tumours are listed in Table 1 All tumours showed a blastomatous or undifferentiated mesenchymal component and an epithelial component The components were mixed and present in varying amounts in different sections of the tumours and showed several types of differentiation and varying degrees of maturation Besides the typical blastomatous component myxoid areas were seen in ten cases and chondroid differentiation in two one of which also revealed extensive bone formation Maturation in the epithelial component appeared as tubular formations in ten of the cases and signs of epidermoid differentiation were seen in seven cases

The variegated histologic spectrum shown by these tumours is illustrated in Fig 1–22 The blastomatous areas consisted of small cells with dark nuclei and a sparse undemarcated light cytoplasm The cells were arranged without orientation and often appeared as cuffs around thin walled endothelial lined vessels (Fig 1) the intermediate areas of a clearly blastomatous areas now appeared epithelial and in some instances were oriented around a central defect or a lumen (Fig 3) Reticulin positive fibers surrounded these small rounded areas but were not seen within (Fig 4) The epithelial component consisted of small cubic to box shaped cells with small dark nuclei and a light rather well demarcated cytoplasm The cells were arranged in branching tubular formations or in solid cords (Fig 2 and 5) In one case there were areas in which the solid cords showed numerous keratin pearl formations (Fig 6) Signs of differentiation in the mesenchymal component were seen as cellular elongation and arrangement in fascicles (Fig 7) or as stellate cells placed in a more abundant alcian positive intercellular ground substance which seemed to point towards myxoid differentiation (Fig 8) Collagenous fibers were sparse as were islands of immature cartilage (Fig 9) and areas with bone formation (Fig 10) Evidence of myofibrils or crossstriation was not found From areas of the above described appearance there were transitions to areas with a marked pleomorphism of the cells

In five cases the tumours had areas of varying size dominated by a single tumour component which looked like a monotypic carcinoma (cases 1 2 and 4) (Fig 11) or a monotypic sarcoma (cases 7 and 10) (Fig 12) A constant feature of the tumours was an abundance of vessels Infiltrative tumour growth within and outside the tumour pseudocapsule appeared as islands of either mesenchymal or epithelial tumour components In only one case (case 1) were such islands of mixed type (Fig. 21) In two cases (cases 2 and 4) the infiltrative tumour growth outside the blastoma was particulary pronounced according to the macroscopic findings In both cases the rounded blastomas were laterally and caudally surrounded by an adenocarcinoma of mixed monomorphic (Fig 13) and pleomorphic appearance (Fig 14) while medially and cranially there were epidermoid carcinomas (Fig 15) of several types and grades of differentiation some areas of which appeared as solid islands (Fig 16) with transitions into a »basaloid« (Fig 17) or cribriform pattern and in others of which the tumour cells were box shaped with a light and well demarcated cytoplasm In both cases there were zones of collision consisting of a *mixture* of infiltrating adenocarcinoma and epidermoid carcinoma

The local recurrences appeared microscopically less differentiated (Fig 18) than the corresponding surgical specimens (*Fig 12*) and they were mainly monotypic either mesenchymal or epithelial

The metastases were also mainly monotypic Within the thoracic cavity they were either *epithe*

TABLE 1 *Pulmonary Blastoma Summary of Clinical and Pathological Findings in 11 Patients Bispebjerg Hospital 1971–1978*

No	Age Sex	Symptoms Duration	Treatment	Primaries: Size	Primaries: Location	Primaries: Epithelial maturation	Primaries: Mesenchymal maturation	Secondaries: Location	Secondaries: (components)	Follow up	(months)	Autopsy
1	59	Pain 3 months	Pneumonectomy Chemotherapy	6 × 5 × 5	RML RLL	Tubular	Myxoid	Hilar nodes Lung pleura Suprarenal gl	(tubular) (mixed) (mesenchymal)	Dead	1	Yes
2	64	Dyspnoe 34 months	Pneumonectomy	5 × 4 × 3 Polypoid	RL	Tubular Epidermoid	Myxoid	Hilar nodes	(tubular)	Dead of postoperative respiratory failure	½	Yes
3	58 ♂	Hemoptysis Cough 2 months	Pneumonectomy Chemotherapy	8 × 4 × 4 Polypoid	RL	Tubular	Myxoid Cartilage	Chest wall	(mesenchymal)	Alive with recurrence	15	Alive
4	66 ♂	Hemoptysis 1 month	Pneumonectomy	10 × 8 × 5	RUL	Tubular Epidermoid	Myxoid	Hilar nodes and recurrence	(tubular)	Dead	12	Yes
5	73 ♂	Cough 1 month	Pneumonectomy	2 × 3 × 3 Polypoid	RUL	Tubular Epidermoid	Myxoid bone Cartilage	Hilar nodes and recurrence	(epidermoid)	Dead	24	Yes
6	67 ♂	Hemoptysis 4 months	Lobectomy	2 × 2½ × 2½	LLL	Tubular Epidermoid	Myxoid	Recurrence	(epidermoid)	Dead	82	Yes
7	69 ♂	Lung infiltrate 1 month	Lobectomy	4 × 5 × 6	RUL	Tubular Epidermoid	Myxoid	Hilar nodes and recurrence	(mesenchymal)	Dead	6	Yes
8	66 ♂	Neurologic 1 month	Craniotomy Chemotherapy Radiation	10 × 8 × 4 Polypoid	LUL	Tubular	Myxoid	Hilar nodes Lung brain Kidney	(tubular) (mesenchymal) (mixed)	Dead 9 months after craniotomy	9	Yes
9	68 ♂	Cough 1 month	None	5 × 5 × 5 Polypoid	LLL	Tubular		Hilar nodes and lung	(tubular)	Dead	½	Yes
10	69 ♂	Lung infiltrate Incidentally	None	10 × 10 × 10	LLL	Tubular Epidermoid	Myxoid	Hilar nodes Lung liver and intestine	(mesenchymal)	Dead 6 months after biopsy	6	Yes
11	77 ♂	Lung infiltrate Incidentally	None	10 × 10 × 10	RLL	Epidermoid	Myxoid	Hilar nodes	(epidermoid)	Autopsy diagnosis	0	Yes

and 11) lung infiltrates were diagnosed during investigation of arteriosclerotic heart disease which made them inoperable

The tumours were right sided in seven cases and left-sided in four In eight cases the tumour was confined to one lobe in one case to two neighbouring lobes and in two cases the tumour involved parts of all three lobes of the right lung

Gross Pathology

The tumours were rounded rather well demarcated and surrounded by a pseudocapsule of compressed lung tissue or bronchial wall which further investigation showed to be invaded by tumour growth The tumour size ranged from 2 5 cm to 10 cm in diameter In five cases (cases 2 3 5 8 9) the tumours showed partly intrabronchial growth part of which appeared as smooth polypoid grape like processes free of the bronchial mucosa In one case (case 10) a similar smooth finger like process was seen in a branch of a pulmonary vein extending into the left auricle of the heart The cut surface of the tumours was smooth and bulging and on inspection was greyish white to pink with scattered haemorrhages and necroses The concistency was rather soft In two cases (cases 2 and 4) a central part of the tumour matched the above description the surrounding tumour tissue being greyish to yellow and rather dry with a plane cut surface

Microscopic Findings

Some histologic features of the tumours are listed in Table 1 All tumours showed a blastomatous or undifferentiated mesenchymal component and an epithelial component The components were mixed and present in varying amounts in different sections of the tumours and showed several types of differentiation and varying degrees of maturation Besides the typical blastomatous component myxoid areas were seen in ten cases and chondroid differentiation in two one of which also revealed extensive bone formation Maturation in the epithelial component appeared as tubular formations in ten of the cases and signs of epidermoid differentiation were seen in seven cases

The variegated histologic spectrum shown by these tumours is illustrated in Fig 1–22 The blastomatous areas consisted of small cells with dark nuclei and a sparse undemarcated light cytoplasm The cells were arranged without orientation and often appeared as cuffs around thin walled endothelial lined vessels (Fig 1) the intermediate areas often showing necroses The reticulin pattern was delicate and indistinct A slight change in this picture was seen as small rounded areas consisting of tumour cells which though of the same size as the clearly blastomatous areas now appeared epithelial and in some instances were oriented around a central defect or a lumen (Fig 3) Reticulin positive fibers surrounded these small rounded areas but were not seen within (Fig 4) The epithelial component consisted of small cubic to box shaped cells with small dark nuclei and a light rather well demarcated cytoplasm The cells were arranged in branching tubular formations or in solid cords (Fig 2 and 5) In one case there were areas in which the solid cords showed numerous keratin pearl formations (Fig 6) Signs of differentiation in the mesenchymal component were seen as cellular elongation and arrangement in fascicles (Fig 7) or as stellate cells placed in a more abundant alcian positive intercellular ground substance which seemed to point towards myxoid differentiation (Fig 8) Collagenous fibers were sparse as were islands of immature cartilage (Fig 9) and areas with bone formation (Fig 10) Evidence of myofibrils or crossstriation was not found From areas of the above described appearance there were transitions to areas with a marked pleomorphism of the cells

In five cases the tumours had areas of varying size dominated by a single tumour component which looked like a monotypic carcinoma (cases 1 2 and 4) (Fig 11) or a monotypic sarcoma (cases 7 and 10) (Fig 12) A constant feature of the tumours was an abundance of vessels Infiltrative tumour growth within and outside the tumour pseudocapsule appeared as islands of either mesenchymal or epithelial tumour components In only one case (case 1) were such islands of mixed type (Fig. 21) In two cases (cases 2 and 4) the infiltrative tumour growth outside the blastoma was, particulary pronounced according to the macroscopic findings In both cases the rounded blastomas were laterally and caudally surrounded by an adenocarcinoma of mixed monomorphic (Fig 13) and pleomorphic appearance (Fig 14) while medially and cranially there were epidermoid carcinomas (Fig 15) of several types and grades of differentiation some areas of which appeared as solid islands (Fig 16) with transitions into a »basaloid« (Fig 17) or cribriform pattern and in others of which the tumour cells were box shaped with a light and well demarcated cytoplasm In both cases there were zones of collision consisting of a mixture of infiltrating adenocarcinoma and epidermoid carcinoma

The local recurrences appeared microscopically less differentiated (Fig 18) than the corresponding surgical specimens (Fig 12) and they were mainly monotypic either mesenchymal or epithelial

The metastases were also mainly monotypic Within the thoracic cavity they were either epithe

TABLE 1 *Pulmonary Blastoma Summary of Clinical and Pathological Findings in 11 Patients Bispebjerg Hospital 1971–1978*

No	Age Sex	Symptoms Duration	Treatment	Primaries: Size	Primaries: Location	Primaries: Epithelial maturation	Primaries: Mesenchymal maturation	Secondaries: Location	Secondaries: (components)	Follow up	(months)	Autopsy
1	59♀	Pain 3 months	Pneumonectomy Chemotherapy	6 × 5 × 5	RML RLL	Tubular	Myxoid	Hilar nodes Lung pleura Suprarenal gl	(tubular) (mixed) (mesenchymal)	Dead	1	Yes
2	64♀	Dyspnoe 84 months	Pneumonectomy	5 × 4 × 3 Polypoid	RL	Tubular Epidermoid	Myxoid	Hilar nodes	(tubular)	Dead of postoperative respiratory failure	½	Yes
3	58♂	Hemoptysis Cough 2 months	Pneumonectomy Chemotherapy	8 × 4 × 4 Polypoid	RL	Tubular	Myxoid Cartilage	Chest wall	(mesenchymal)	Alive with recurrence	15	Alive
4	66♂	Hemoptysis 1 month	Pneumonectomy	10 × 8 × 5	RUL	Tubular Epidermoid	Myxoid	Hilar nodes and recurrence	(tubular)	Dead	12	Yes
5	73♂	Cough 1 month	Pneumonectomy	2 × 3 × 3 Polypoid	RUL	Tubular Epidermoid	Myxoid bone Cartilage	Hilar nodes and recurrence	(epidermoid)	Dead	24	Yes
6	67♂	Hemoptysis 4 months	Lobectomy	2½ × 2 × 2½	LLL	Tubular Epidermoid	Myxoid	Recurrence	(epidermoid)	Dead	82	Yes
7	69♂	Lung infiltrate 1 month	Lobectomy	4 × 5 × 6	RUL	Tubular Epidermoid	Myxoid	Hilar nodes and recurrence	(mesenchymal)	Dead	6	Yes
8	66♂	Neurologic 1 month	Craniotomy Chemotherapy Radiation	10 × 8 × 4 Polypoid	LUL	Tubular	Myxoid	Hilar nodes Lung brain Kidney	(tubular) (mesenchymal) (mixed)	Dead 9 months after craniotomy	9	Yes
9	68♂	Cough 1 month	None	5 × 5 × 5 Polypoid	LLL	Tubular		Hilar nodes and lung	(tubular)	Dead	½	Yes
10	69♂	Lung infiltrate Incidentally	None	10 × 10 × 10	LLL	Tubular Epidermoid	Myxoid	Hilar nodes Lung liver and intestine	(mesenchymal)	Dead 6 months after biopsy	6	Yes
11	77♂	Lung infiltrate Incidentally	None	10 × 10 × 10	RLL	Epidermoid	Myxoid	Hilar nodes	(epidermoid)	Autopsy diagnosis	0	Yes

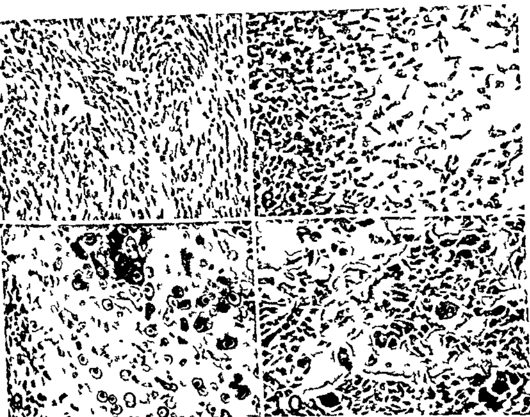


Fig 7 10 Pulmonary Blastoma proper mesenchymal d fferent at on
7 Fascicles of elongated mesenchymal tumour cells a few show ng mitotic act vity (Case 9 autopsy specimen)
8 Myxo d d fferentiation Stellate cells in an abundant myxomatous groundsubstance (Case 3 surgical specimen)
9 Chondromatous differentiation Edge of island of fetal type cart lage (Case 5 surgical spec men)
10 Osteo d d fferentiat on Osteoclasts and osteoblasts lining bony trabeculae (Case 5 surg cal spec men)

ial (Fig 19) or mesenchymal while extrathoracic metastases were mesenchymal of appearance (Fig 20) In only two cases (cases 1 and 8) were solitary metastases of mixed type found (Fig 21 and 22)

Treatment and Follow up

In seven patients a resection was performed of these six had a radical resection while in one (case 1) tumour tissue in the chest wall and diaphragm remained In three cases (cases 1 2 and 4) lymph node metastases were resected as well

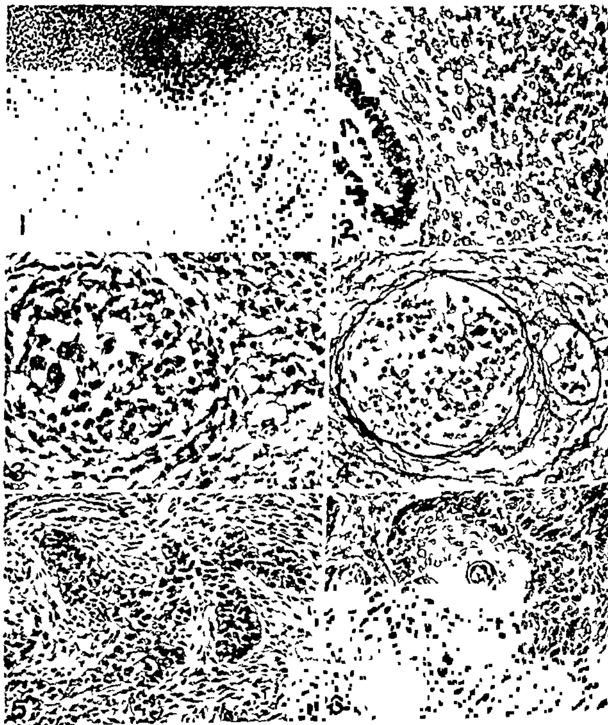
Of these seven patients two died shortly after the operation one because of rapid tumour dissemination (case 1) and one because of an impaired lung function Four survived respectively 6 12 24 and 82 months and at autopsy all four showed recurrent tumour tissue and/or metastases One patient (case 3) was still alive 15 months postoperatively with a histolog c recurrent tumour in the chest wall

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In the three year period 1973 through 1975 1185 cases of malignant primary lung tumours were histologically diagnosed at the department of pathology Bispebjerg Hospital In Table 2 these cases are shown according to the histologic type Six of our cases of PB were diagnosed in the same period The frequency of PB thus was 0 5 per cent of all primary malignant lung tumours

DISCUSSION

The diagnosis of the 11 cases of PB presented here was based on the characteristic and widely accepted microscopic appearance a mixture of immature mesenchymal and epithelial components The differential diagnosis would involve distinguishing other lung tumours of mixed type Tumours as epider



Original magnification 160 and hematoxylin eosin stain unless otherwise noted

Figs 1–6 Pulmonary Blastoma (PB) proper epithelial differentiation
1 Most immature and most characteristic feature of a PB as registered in all the presented cases Undifferentiat tumour cells seen as cuffs around small vessels The cuff in the lower right corner reveals the earliest possil epithelial like differentiation (Case 11 autopsy specimen × 63)
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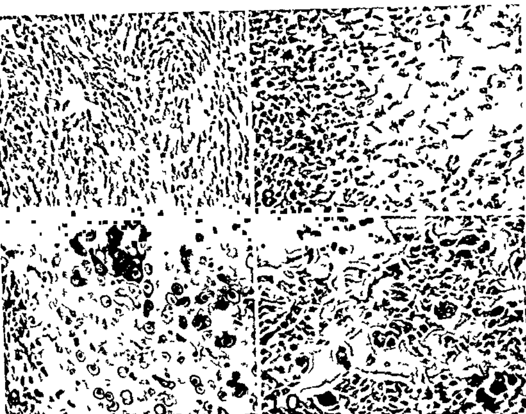


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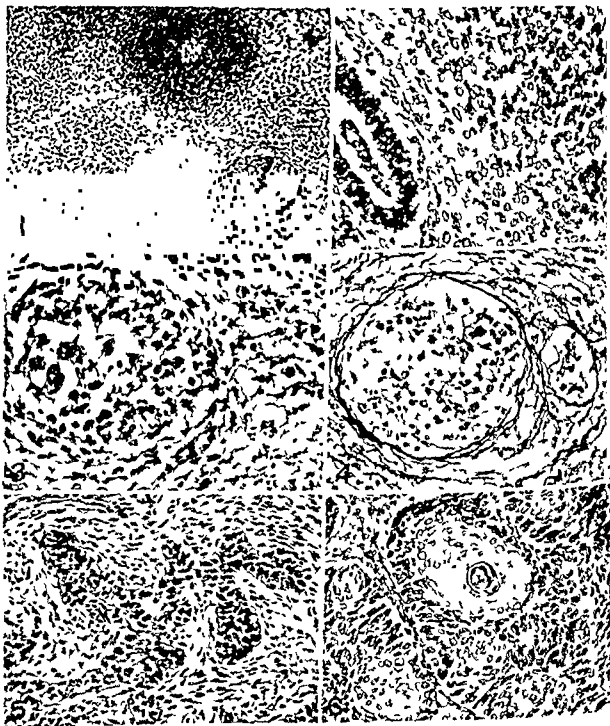
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2 Classic PB An undifferentiated immature stroma and part of tubular formation resembling fetal lung tissue *12* weeks gestational age (Case 2 surgical specimen)
3 Two islands of small tumour cells of epithelial like appearance (Case 8 autopsy specimen)
4 Same area as Fig 3 Reticulin positive fibers surround the two islands (Case 8 autopsy specimen Silver impregnation)
5 Obvious epithelial differentiation Small immature box shaped epithelial cells arranged in branching cords two of which show necrotic centers as to beginning of tubular formations (Case 6 surgical specimen)
6 Epidermoid differentiation Well demarcated cords of small immature epithelial cells In one cord abrupt differentiation into a keratin pearl is seen (Case 5 surgical specimen)

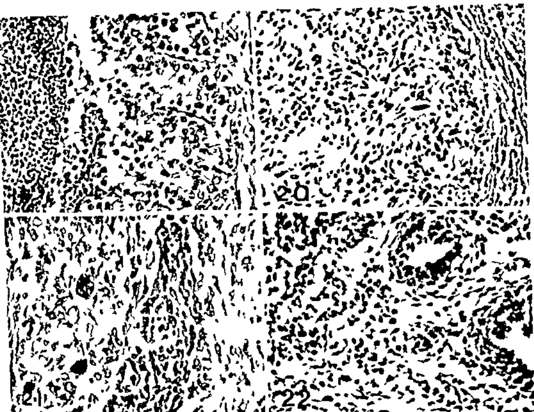


Fig 19–22 Pulmonary Blastoma different types of appearance in the metastases

19 Lymph node metastasis Box shaped tumour cells with light cytoplasm The tumour cells arranged in adenomatous formations appearing in the marginal sinus of a hilar lymph node Other types of epithelial differentiation in this case are shown in Fig 14 and 15 (Case 4 surgical specimen)

20 Liver metastasis Elongated tumour cells of monotypic mesenchymal appearance (Case 10 autopsy specimen)

21 Satellit tumour of the lung Island of epithelial tumour cells surrounded by a very pleomorphic mesenchymal tumour component Same case as Fig 11 (Case 1 surgical specimen)

22 Biphasic differentiation in metastatic lesion of the kidney Several tubular formations in an undifferentiated stromal component (Case 8 autopsy specimen)

Fig 11–18 Pulmonary Blastoma different types of monotypic differentiation

11 Monomorphic adenocarcinoma Small box shaped cells with dark nuclei and a light well demarcated cytoplasm (Case 1 surgical specimen)

12 Monomorphic sarcoma Fascicular arrangement of spindle cells The nuclei are enlarged and there are several mitoses (Case 7 surgical specimen)

13 Monomorphic adenocarcinoma Small undemarcated cylindrical cells with vesicular nuclei Crowding of cells (Case 2 surgical specimen)

14 Polymorphic adenocarcinoma (Mature type) (Case 4 surgical specimen)

15 Epidermoid carcinoma Same case as Fig 14 Typical epidermoid differentiation of tumour showing keratinisation and cellular pleomorphism (Case 4 surgical specimen)

16 Undifferentiated carcinoma Well demarcated islands of undifferentiated monomorphic epithelial tumour cells (Case 2 surgical specimen)

17 »Basalioma« like area Same case as Fig 2 13 and 16 (Case 2 surgical specimen)

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TABLE 2 *Histologic Type Distribution (WHO classification) in 1185 Cases of Primary Lung Cancer Diagnosed at Bispebjerg Hospital 1972–1975*

Sex	Number of cases	Epidermoid carcinoma Per cent	Small cell carcinoma Per cent	Adeno carcinoma Per cent	Large cell carcinoma Per cent	Others Per cent	Pulmonary blastoma Per cent
♀	240	32,9	20 4	27,5	12,5	6 3	0 4
♂	945	48,9	23,5	11,4	13,3	2,4	0 5
Total	1185	45,7	22,7	14,7	13,2	3,1	0 5

moid carcinoma with a spindle cell component, mixed mucosa gland tumours and teratomas should be reasonably easy to distinguish Difficulties, however, may arise in separating PB from certain cases of mesothelioma, hamartoma and carcinosarcoma Transitions between these latter mentioned tumour types and PB have been described (7, 16, 23)

To our knowledge as of 1978, 65 cases of PB had been reported (10) The mean age in the series presented was rather high (11) One of our patients had had symptoms of long duration before treatment Similar long duration of symptoms were reported in the cases of *Bauermeister et al* (1966) and *Parker et al* (1966) Except for a rather high mean age, a high frequency of intrabronchial tumour growth and a high frequency of metastases, our cases did not differ from reported cases with regard to size, location or frequency of local recurrences of the tumour or duration of survival (11) Microscopically we found a high frequency of cases with signs of epidermoid differentiation a component which has only recently begun to be accepted in PB (6, 7, 13, 22 23 29)

Extensive growth of an adenocarcinoma and an epidermoid carcinoma in close contact with a PB as seen in two of our cases have not been previously described Collision of different types of malignant tumours in these cases could be a possible explanation for this The variegated microscopical appearance of the carcinomas being different from ordinary primary bronchogenic carcinomas, however, points towards all the tumour tissue components originating from the blastomas

In all the cases presented here monotypic secondaries were found, metastases of mixed type having been recorded in only two cases This is in contrast to reported cases where most of the histologically described metastases were of mixed type

The prognosis in our cases was poor, within two years nine patients died as a result of their tumour, and one died seven years after resection with a large recurrent tumour A similar case of long term survival ending with death of recurrent tumour was reported by *Kern et al* (1976) Cases of tumour free long-term survival were reported by *Barnard* (1952), *Kennedy & Prior* (1976) and *Iverson & Strachley* (1973)

Some authors regard the PB as a subgroup of carcinosarcoma (18, 23, 27) while others regard the PB as a separate entity (14, 19, 20 21, 24) developed from a pluripotent cell of a single germ layer, here the pulmonary blastema of the mesoderm (25, 26) Mesodermal mixed tumours however, are widely accepted in several other organ systems They all consist of a mixture of mesenchymal and epithelial tumour components showing varying differentiation and maturation although monotypic cases have been recorded (4, 5, 9) Unlike in teratomas, heterotopic components are not found in PB

The etiology of neoplastic growth from the pluripotent cells is unknown Some of the differences in structural appearance and biological behaviour of these mixed tumours might be explained by the primary cellular change, the location of the cell and the induction from the surrounding tissue

The secondaries in our material only occasionally were of the mixed type The usually monotypic sarcomatous or carcinomatous appearance of the secondaries lent support to the hypothesis that in the blastoma one or several transformations had taken place resulting in a local dominance or metastatic growth of the transformed tumour components which in some instances were more anaplastic in appearance

Supported by the reports of transitions between blastoma and carcinosarcoma (7 23) and by the reports of morphological similarities between fetal lung tissue hamartoma and blastoma (11, 16, 26 28) our opinion is that these three tumours are mixed mesodermal tumours originating from the same cell type Differences in morphology and biological behaviour may be explained by the type of cellular defect produced by the initiating factors

The 11 cases presented here constitute the largest material of PB diagnosed in a single department To date *Davis et al* (1972) reported five cases and in the 50 year period 1915–1965 *Stackhouse et al* (1969) found only four from the Mayo Clinic Six of our cases were diagnosed in a three year period during which 1 185 cases of primary malignant lung tumour were diagnosed at our department The frequency of PB thus was 0 5 per cent of all malignant lung tumours

A possible explanation for the high number of cases diagnosed at our department might be related to our particular interest in this type of tumour and the search that we therefore directed specifically towards it combined with the fact that the danish autopsy regulations data registry health service system and health service contact pattern help to facilitate the follow up in a single department of a patients course through biopsy treatment and ultimately autopsy

A preoperative diagnosis of PB is very difficult to establish The presence of both mesenchymal and epithelial tumour cells or tumour tissue in the preoperative microscopic specimens are highly suggestive of a mixed tumour hence PB (8) Further examples of this tumour might be disclosed among cases of lung cancer with secondaries of divergent microscopic appearance PB is considered a very rare tumour but the results of this survey would indicate that it is remarkably more common than previously believed

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OSTEOGENIC SARCOMA OF THE BREAST

Report of a Case

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Benediktsdottir K Lagerberg F Lundell L & Thulin A Osteogenic sarcoma of the breast Report of a case Acta path microbiol scand Sect A 88 161–165 1980

The clinical mammographical cytological and histological features of a mammary osteogenic sarcoma are reported The tumor which was removed by only local excision had suddenly appeared in the same breast from which previously an intraductal papilloma had been removed Nearly three years after the operation there is no indication of recurrence

Key words Breast osteogenic sarcoma

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Extra skeletal bone formation may be formed under a variety of different conditions The occur rence of true osteogenic sarcoma outside the skeleton is however rare Extra skeletal osteogenic sarcomas have been described for instance in the kidney the thyroid gland the urinary bladder the soft tissue of the extremities and in the breast (*Fine & Stout* 1956 *Jernstrom et al* 1963 *Rao et al* 1978) *Bonet* (1700) may have been the first to observe a ›bony‹ tumor in the female breast However *Lancereaux* (1860) gave the first descrip tion of the histological picture of a breast tumor consisting of osteoid tissue

Sarcoma of the breast may constitute between 0 2–3% of all breast malignancies These neo- plasms are a heterogenous group of mesenchymal tumors and may to different extents contain bone cartilage fibrinous and osteoid tissue Breast sarcomas with osteoid components have been presented as teratoid mixed tumors (*Mc Iver* 1923) osteoid sarcoma (*Rottino & Howley* 1945) osteo chondro[illegible] 1943) or [illegible] 1963 *Sun* [illegible]se discre pa[illegible]cies regarding the nomenclature may to some degree account for the difficulties encountered in the estimation of the true incidence of osteogenic sarcoma in the mammary gland Furthermore *Rottino and Howley* (1945) pointed out that the osteoid component could easily be overlooked and be found first in a secondary tumor Still *Jernstrom et al* (1963) reported the incidence of osteogenic sarcoma to be about 10% of all sarcoma of the breast

In this report we present the clinical mammo graphical cytological and histological features of a true mammary osteogenic sarcoma

CASE HISTORY

A previously healthy 72 year-old woman attended the out patient clinic in November 1968 with a tumour in the upper lateral quadrant of the left breast She had been aware of a lump for 2 months No clinical sign of malignancy was found A fineneedle aspiration biopsy revealed an intraductal papilloma a diagnosis later confirmed by focal excision The lesion was however interpreted to be of a pre-cancerous nature but had been radically removed The patient was followed up at the



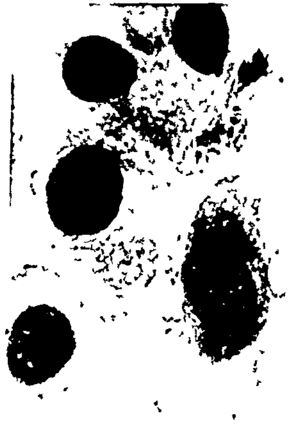


Fig 1 Mammography demonstrating a b cyclic calcified tumor in the upper part of the breast

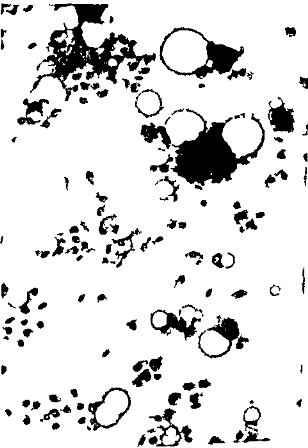




Fig 2 The cytological appearance of the tumor (a) The two cell types epithelial like spindle cells and multinucleated giant cells (MGG ×25) (b) Spindle cells one showing mitotic figure (MGG high power field) (c) Multinucleated giant cell (MGG high power field)

out patient clinic until 1971 with no indication of recurrence

In december 1976 she returned to the Department of Oncology with a tumor in about the same place as the one previously excised The lump measured about 3 cm in diameter and was not fixed to the surrounding structures The tumor was irregular in shape but well circumscribed She had noticed this tumor for a couple of weeks and she presented no concominant breast complaint. There was no history of nipple discharge and no discolouration of the skin Mammographic investigation (Fig 1) revealed a bicyclic tumor with calcifications particularly in the walls of the cystic formations The tumor was not considered malignant Fineneedle aspiration was performed The smears were difficult to interpret (see below) but the picture could be consistent with a malignant tumor Owing to the discrepancy between the two pre operative investigations a frozen section procedure was carried out with the patient under general anesthesia The pathologist reported a mesenchymal tumor with osteoid but no further classification could be made The wound was closed and the post operative course was uneventful The patient refused any further operation and she has since then been followed up at the out patient clinic Two subsequent ^{99m}Tc d phosphonate skeletal scintigraphies have not revealed any primary tumor in the skeleton or a dissemination of the disease Repeated X ray surveys of the skeleton and the lungs have also been normal In September 1979 the patient is free from any symptom indicating recurrence and /or metastases from the sarcoma

Pathology

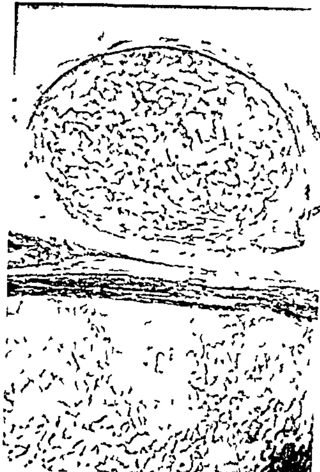
The fine needle aspiration biopsy from the lesion revealed a very cellular material with two cell types mostly dissociated (Fig 2) The more common type was moderately large and epithelial like with abundant cytoplasm and rather big nucleus with enlarged and prominent nucleolus Furthermore many mitotic figures were seen The nucleus often lay excentric in the cell The other type was a giant cell with granulated cytoplasm containing small round nuclei This unusual cytological picture suggested malignancy

The specimen obtained by local excision consisted of breast tissue with a globular tumor that measured 4 × 5 × 3 5 cm and was well circumscribed but unencapsulated (Fig 3) At the margins small knobs were seen protruding and pushing their way into the surrounding fat The cut surface was grey homogenous firm and gave a gritty sensation suggesting the presence of bone Frozen sections showed a mesenchymal tumor with areas of osteoid which could not be properly classified Paraffin sections showed a very cellular tumor uniform in appearance (Fig 4) The tumor was characterized by



Fig 3 The cut surface of the tumor with a sharp conture at the periphery and whitish calcified areas

types of cells The cells forming the bulk of the tumor were large spindle cells displaying a varying degree of pleomorphism with reticular type of nuclei mostly excentric The cells were grouped in intertwining whorling bundles Many cells were undergoing mitotic division In high power field (HPF) one could find in the most cellular areas four mitotic figures at the most Interspersed among these cells often in close contact with the osteoid were giant cells containing many small deeply-staining nuclei In some areas there was much osteoid substance partly mineralized but other areas were purely cellular Between the cells there was a delicate fibrous stroma and scattered blood vessels Accordingly the tumor had the character of a pure osteogenic sarcoma indistinguishable from osteogenic sarcoma of skeletal origin Surrounding the tumor mass there was fat with strands of breast tissue with small ducts No evidence of intraductal papilloma or fibroadenoma was found in the sections Furthermore no cartilage was seen In ... tumor th ... tissue b ... free marg ...



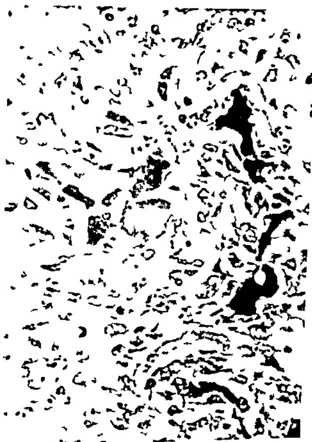


Fig 4 (a) Knobbing at the marg ns of the tumor A of dark stained osteoid separated by cellular sheets Cellular area with closely packed pleomorph c sp cells multinucleated giant cells and osteoid tissue (H × 25)

DISCUSSION

Osteogenic sarcoma of the breast is a disease adult and old age and has only once been describ in a male breast (*Sun* 1952) The time betwe discovery of the tumor and the medical attent varies between a month in our case up to 30– years (*Rottino & Howley* 1945 *Sun* 1952) The seem to be no characteristic features of osteoger sarcoma that clinically distinguish them from oth malignant breast tumors

It is difficult to gain an insight into the cl n c behaviour of specific histological types of brea sarcomas owing to their uncommon occurrenc and the confusion in terminology There ar however indications that malignant bone and/c cartilage in a breast sarcoma is a sign of poc prognosis (*Smith & Taylor* 1969 *Barnes & Pie trus*ka* 1977) Accordingly osteogenic sarcoma o the breast are considered highly malignant ofte rapidly growing giving early local recurrence an metastases by the hematogenous route to primarily the lungs but metastases in the brain and kidney ar also known (*Rottino & Howley* 1945 *Rott no & Willson* 1945 *Sun* 1952 *Wester & Finlay Jon s* 1960 *Jernstrom et al* 1963 *Smith & Taylor* 1969) Lymphatogenous spread is rare and axillary metastases have only occassionally been described An adequate surgical treatment would thus be a simple mastectomy There is no information available that describes any positive effect of adjuvant therapy such as radiotherapy and/or cytotoxic drugs

There are certain microscopic features of the tumor that have been discussed in relation to the prognosis of the patients Although *Allan and Soule* (1971) found no histological difference between tumors with rapid and prolonged courses others have claimed that the prognosis rests upon the number of mitotic figures per HPF and how the tumor behaves towards the surrounding breast tissue as well as the cellular atypia and tumor size (*Norris & Taylor* 1968 *Barnes & Pietruszka* 1977) *Tumors that had less than 5 mitoses per HPF* and pushed their margins were suggested to have better prognosis than those with 8–10 mitoses per HPF and infiltration into the breast tissue According to these criteria the sarcoma of our patient would have

a favourable prognosis which the clinical course hitherto would also suggest

Efforts to explain the ethiology of breast sarcoma in general and osteogenic sarcoma in particular have given rise to a number of different theories through the years The histogenesis is however still not settled The concept most generally held is that these tumors arise through metaplasia and malignant change in stromal cells (mesenchymal connective tissue cells) considered to be totipotential being able to differentiate into various directions under the proper environmental stimulus (*Das Gupta et al* 1968) Another theory is misplacement of pluripotential embryonic cells during development much like a teratomatous growth (*Mc Iver* 1923) Others have observed an intimate association between osteogenic sarcoma and fibroadenoma (*Rottino & Howley* 1945 *Curran & Dodge* 1962 *Jernstrom et al* 1963) *Smith and Taylor* (1969) described two patients with mixed mesenchymal tumors who had previously been operated for intraductal papilloma in the same area and five other cases having intraductal papillomas within ducts adjacent to the tumor In the present case there was no evidence of fibroadenoma in either the first or the second specimen and no residue of intraductal papilloma could be found in the tissue around the sarcoma It should however be pointed out that previously an intraductal papilloma had been removed from almost the same place in which the osteogenic sarcoma later occurred

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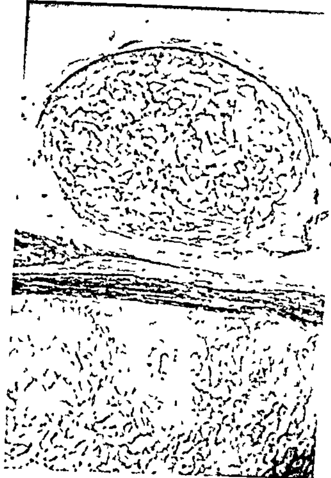
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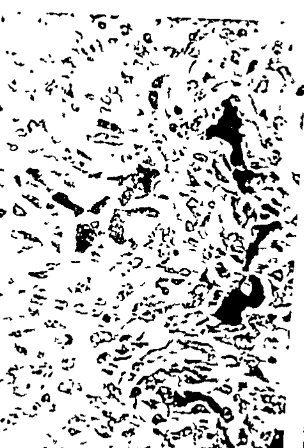


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CORONARY ATHEROSCLEROSIS IN MIDDLE-AGED AND ELDERLY FINNISH MEN

PATHOLOGICAL STUDY OF CORONARY ARTERIES IN VIOLENT AND SUDDEN CORONARY DEATHS

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Rissanen Viljo Coronary atherosclerosis in middle aged and elderly Finnish men Pathological study of coronary arteries in violent and sudden coronary deaths Acta path microbiol scand Sect A 88 167–173 1980

The occurrence of coronary atherosclerosis was studied in 271 Finnish men aged 40 years or more of whom 143 had met a violent death and 128 had died suddenly of coronary heart disease (CHD) A coronary stenosis was found in half of the men who had met a violent death and were over 50 years old The occurrence of the stenosis was closely related to the extent of the raised atherosclerotic lesions (RL) and to the degree of the plaque calcification In 60 % of the sudden deaths (SD) the extent of RL did not differ from that in violent deaths The stenosis patterns in these SD were similar to those in living CHD patients The remaining SD had an extreme stage of coronary artery involvement, i e extensive plaques with severe obstructive changes The men in this study series of violent deaths born in various parts of the country did not differ with regard to the extent of RL Within the upper half of the RL area range men born in the east were consistently shown to have a stenosis while the occurrence in men from the western counties was more random Severe stages of coronary artery involvement tended to be more frequent in SD patients born in the east than in those born in the west It was concluded that there are probably two background factors concerning the difference in CHD mortality between men in western and eastern Finland 1) the tendency to form a stenosis by the advancement of RL extent is stronger and 2) the progressive development of coronary artery disease in CHD patients is more frequent in the east than the west

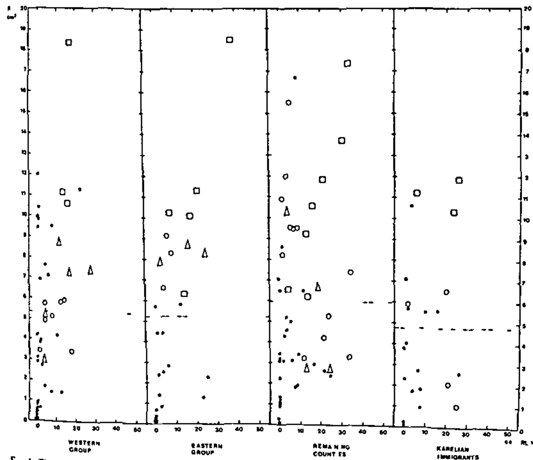
Key words Atherosclerosis coronary artery coronary heart disease Finnish men

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Patho anatomic studies on coronary atherosclerosis have not revealed a clear explanation for the regional difference in the mortality from coronary heart disease (CHD) in Finland The extent of coronary plaques seems to be similar in men from eastern and western Finland (*Rissanen* 1974) although the mortality rate of coronary heart disease is clearly higher in the former than the latter (*Valkonen & Niemi* 1978) Calcific degeneration of the plaques does however seem to occur earlier and be more extensive in men from eastern Finland (*Mottonen* 1969 *Rissanen* 1974)

In the present study coronary atherosclerosis was studied in a Finnish autopsy series of violent deaths and in a series of sudden coronary deaths The risk population with regard to coronary artery involvement was determined on the basis of the comparison of these two series Particular interest was focused on coronary lesions causing stenosis Men born in various regions of the country were compared in order to formulate a theory for atherosclerosis which could explain the regional differences in CHD mortality



Fg 1 The area of raised lesion (cm²) in relation to the degree of calcification (cal /RL %) and the occurrence of d fferent stenosis patterns in birthplace groups of violent deaths The dotted line indicates the mean of the RL

□→tr ple vessel disease	stenosis in 3 coronaries
Δ→double vessel disease	stenosis in 2 coronaries
O→single vessel disease	stenosis in 1 coronary artery
•→no stenosis	

and 55% of the violent deaths the extent of RL exceeded 4 cm² The prevalence of single vessel d sease was the same (27%) in SD with a normal extent of RL i e below 12 cm² as in violent deaths (26%) with an RL of 4–12 cm² (Table 1) Double and triple vessel diseases were twice as common in the former (22% and 42%) as in the latter (12 5% and 19 5%) Taking into account the extent of RL and the type of the stenosis pattern it was estimated that about 40% of the sudden deaths could be used to form a random sample of men with a stenosis from the population The remaining SD victims had a severe stage of coronary artery involvement i e extensive plaques and/or severe obstructive changes

Calcifications were found in all violent deaths with a coronary stenosis (Fig 1) in 90% of men with no stenosis but with an RL over 4 cm² and in 54% of men with no stenosis and an RL below 4 cm² The degree of calcific degeneration of the plaques was most advanced in men with triple vessel disease (cal /RL 15 2 ± 6 6%) or double (cal /RL 14 3 ± 8 9%) vessel disease (Fig 1) Also th[illegible] tion w[illegible] more a[illegible] cases without stenosis (cal /RL 4 6 ± 5 6%) even if the area of the RL was similar (4–12cm²) (t test p < 05)

Regional Differences

In the study series of violent deaths the mean extent and range of the RL were the same in men from all the regions (Fig 1) The area of the RL in

MATERIAL AND METHODS

Violent deaths The series consists of 143 men aged 40 to 78 years who met a violent death i e accident suicide or homicide The autopsies were performed during a period of 12 months at the Department of Forensic Medicine of the University of Helsinki

Coronary deaths The series comprises 128 men aged 40 to 77 years who died suddenly within 24 hours after the onset of the fatal attack The original series included 140 men collected from among victims classified as coronary deaths at routine medico legal autopsies per formed during a ten month period at the Department of Forensic Medicine of the University of Helsinki The autopsy rate of prehospital sudden deaths (SD) is about 80% in Helsinki All such autopsies are carried out at the Department of Forensic Medicine In the analysis twelve cases were excluded from the original series for the following reasons five men were younger than 40 years two men were born abroad and were not Finns the birthplace of one man was unknown and in four men whose death was unwitnessed definite proof of a CHD death was missing and other causes of death were possible An acute myocardial infarction (AMI) was revealed in 49% of the 128 men accepted in the series In the remaining cases the diagnosis of a CHD death was based on the disease history history of the fatal attack autopsy findings of the heart and exclusion of other causes of death

Place of birth

On the basis of birthplace the individuals of the two series were divided into four groups

Western group Men born in the present four western counties of Finland (Åland Turku and Pori Hame Vaasa) where the mortality rate of CHD is the lowest (*Pyorala* 1974) The group included 43 violent and 28 sudden deaths The mean age in the former case was 56 0 and in the latter 57 3 years

Eastern group men born in the present five counties in the eastern and central parts of the country (North Carelia Kuopio Mikkeli Oulu Central Finland) where the mortality rate of CHD in men is the highest (*Pyorala* 1974) There were 27 men in both series born in these counties the mean age in cases of violent death being 53 2 and of sudden death 55 9 years

Remaining provinces Men born in the three remaining counties (Uusimaa Kymi Lapland) where the rate of CHD mortality lies between that of the western and eastern counties (*Pyorala* 1974) The group included 51 cases of both violent and sudden deaths The mean age of the former was 56 0 and of the latter 56 4 years

Carelian immigrant group Men born in areas ceded to the Soviet Union in 1944 There were 22 men in both series born in these areas the mean age in cases of violent death being 53 8 and of sudden death 53 4 years The immigrants have become integrated with the rest of the population and no reliable data specifically concerning their rate of CHD mortality are available

Estimation of the Severity of Coronary Atherosclerosis

The principles developed and used in international studies on atherosclerosis (*Guzman et al* 1968) were followed in the present study The methodological detail have been described in previous papers (*Rissanen* 1972 *Rissanen & Pyorala* 1972) Arterial specimens were prepared from the longitudinally opened right coronary artery and left anterior descending and left circumflex coronary arteries The arterial specimens were fixed in 10% formalin The specimens were radiographed for the exact determination of calcification

The specifications set down by the study group of the International Atherosclerosis Project (*Guzman et al* 1968) were followed in the definition of atherosclerotic lesions *Raised lesion* was a firm elevated intimal lesion with or without other changes covering it or underlying it

The areas of the atherosclerotic lesions measured in square centimetres were assessed from the arterial specimens by the point counting technique (*Rissanen* 1972 *Rissanen & Pyorala* 1972) The degree of coronary calcification was measured from the radiographs of the specimens by applying the point counting technique As shown in the previous papers (*Rissanen* 1972 *Rissanen & Pyorala* 1972) the reproducibility of the results obtained by the point counting technique was good and the accuracy comparable to that obtained by planimetry

The degree of the coronary stenosis was estimated visually in the arterial specimens (*Rissanen* 1972) A stenosis of 50% or more was regarded as significant The sites of occlusions or near occlusions were also recorded The reproducibility of the estimation of stenosis was rather good (*Rissanen* 1972) The prevalences of coronary stenosis obtained by this visual estimation in the SD series were very similar to those obtained in another SD series studied by postmortem coronary angiography (*Rissanen et al* 1978) as well as in an autopsy series of AMI hospital deaths studied using cross sections of the coronary arteries (*Rissanen* 197?) (Table 1)

RESULTS

Coronary atherosclerosis in the total series of violent and sudden deaths

A 50% stenosis of a coronary artery was found in half of the violent deaths over the age of 50 years A stenosis was rare (14%) if the extent of RL was below 4 cm² (Table 2) but it was found in nearly half of the men with an RL of 4–8 cm² and in three out of four men with a larger lesion The upper limit of the range of the RL area seemed to be constant at about 12 cm² (Fig 1) There were only a few cases above this level

Although the mean extent of the raised lesion was twice as large in sudden deaths (SD) as in violent deaths (Fig 2) marked overlapping between the two series was found In 95% of the sudden deaths

TABLE 1 *The Percentages of Different Stenosis Patterns in Violent and Sudden Deaths According to the Extent of Raised Lesion (RL) Compared with the Corresponding Prevalences in Two Postmortem and Two Clinical Series*

Series	Triple vessel disease* (%)	Double vessel disease* (%)	Single vessel disease* (%)	No stenosis (%)
Present series				
Violent deaths (RL 4–12 cm²)	19 5	12 5	26	42
Sudden deaths (RL > 12 cm²)	69	25	6	–
Sudden deaths (RL < 12 cm²)	42	22	27	9
Other postmortem series				
Sudden deaths studied by coronary angiography (Rissanen et al 1978)				
with an old MI	69	28	2	1
without an old MI	37	37	23	3
AMI deaths at hospital (Rissanen 1979)	50 5	35	11 5	3
Clinical series of coronary arteriography				
CHD patients in Helsinki (Hekali 1976)	31	41	28	
CHD patients in the USA (Fuster et al 1975)				
angina pectoris	31	37	24	
subendocardial MI	42	40	13	
transmural MI	38	30	27	

*) ≧ 50 per cent stenosis

TABLE 2 *The Occurrence of Different Stenosis Patterns in Violent and Sudden Deaths in Relation to the Extent of Raised Lesion (RL)*

Series	Number of cases	Triple vessel disease		Double vessel disease		Single vessel disease		No stenosis	
Violent deaths		cases	%	cases	%	cases	%	cases	%
RL > 12 cm²	6	4	66	–	–	1	17	1	17
RL 8 12 cm²	32	11	34 5	4	12 5	8	25	9	28
RL 4 8 cm²	40	3	7 5	5	12 5	11	27 5	20	52 5
RL < 4 cm²	65		–	3	5	6	9	56	86
Total	143	18	13	12	8	26	18	87	61
Sudden deaths									
RL 16 [illegible]0 cm²	1[illegible]	8	67	3	25	1	8		–
RL 12–16 cm²	39	27	69	10	26	2	5		–
RL 8–12 cm	39	20	51	9	23	7	18	3	8
RL < 8 cm²	38	12	32	8	21	14	37	4	10
Total	128	67	52	30	23	24	19	7	6

numbers of sudden and violent deaths was 2 3 in the present western group but 1 1 in the eastern group The numbers of violent deaths probably reflect the ratio of men in Helsinki born in the west and east of the country On the other hand the appearance of sudden deaths in the series reflects the CHD mortality rates in these sub-populations The ratio of the mortality rates of CHD in the sub-populations in Helsinki originating from the [illegible] [illegible] can be

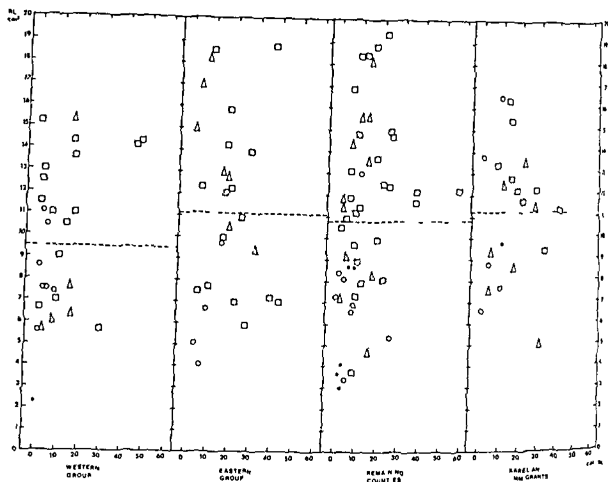


Fig 2 The area of raised lesion (cm²) in relation to the degree of calcification (cal /RL %) and the occurrence of different stenosis patterns (symbols similar to those in Figure 1) in birthplace groups of sudden deaths The dotted line indicates the mean of the RL

the upper range (6–12 cm²) was consistently associated with a stenosis in men born in eastern Finland In the other groups a stenosis was more randomly related to the ectent of RL particularly in men from the western counties (Fig 1) The difference between the eastern and western groups was statistically significant (hypergeometric distribution test $p < 01$) As mentioned above a portion of the series of sudden deaths overlapped with violent deaths with an RL of 4–12 cm² In these violent deaths the prevalence of a 50 % stenosis was lower in the western group (48 %) and immigrants (45 %) than in the eastern (71 %) and remaining (67 %) groups (Fig 1) The geographical groups of violent deaths did not differ significantly with regard to the degree of calcification (cal /RL western men 8 4 ± 7 6 % eastern men 10 1 ± 7 7 %)

Severe coronary atherosclerosis exceeding the degree which commonly seemed to occur in the population (RL over 12 cm²) was less frequent in the western group of sudden deaths (29 %) than the other groups (eastern 48 %, remaining 39 % immigrants 45 %) (Fig 2) There were no significant differences in the prevalences of different stenosis patterns i e triple double or single vessel disease between SD victims born in various geographic regions A severe triple vessel disease i e an occlusion or multiple 50 % stenosis in all three coronaries was however most frequent in the eastern SD group (41 %) least frequent in the western SD group (18 %) and of intermediate frequency in the remaining (24 %) and immigrant (27 %) groups

DISCUSSION

It has been shown that people moving to Helsinki from various counties retain the same rate of mortality from CHD as those staying in their home counties (*Karpela* 1960) At the time of this study the ratio of the rate of CHD mortality between western and eastern men living in the counties was 2 3 (400 617/100 000) in the 35–64 year age group (*Pyorala* 1974) The ratio between the

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expected to be comparable with that in men living in the counties

The risk of CHD is determined by the development of a stenosis in the coronary arteries This seems to be related to both the extent of the plaques and their degree of degeneration The risk of a stenosis and at the same time the risk of a coronary death begins to increase when the area of raised atherosclerotic lesions exceeds about 4 cm² in the coronary artery tree On the other hand there may be a critical upper level in the extent of coronary raised lesions (about 12 cm²) related to vitality (*Rissanen* 1979) persons with a more extensive coronary artery involvement tend to disappear from the population For instance in the present series of violent deaths which represented a sample of the population the RL exceeded 12 cm² in only 4 % of cases

In about 60% of the SD victims the area of raised lesion did not differ from that in the violent deaths The occurrence of different stenosis patterns in these SD seems to correspond to figures reported in living CHD patients studied by clinical coronary arteriography (*Fuster et al* 1975 *Hekali* 1976 Table 1) This portion of the coronary deaths thus seems to form a sample of clinical patients or in part (40%) a sample from among the large number of men with a stenosis in the population Epidemiological studies have shown that most cases of CHD of the community occur among the majority of individuals at moderate risk and only a minor proportion among the few clearly high risk people (*Rose* 1976) Correspondingly it is obvious that a great proportion of coronary deaths occur among the majority of individuals with moderate coronary lesions On the basis of the vital statistics (*Pyorala* 1974) it can be calculated that about half of those with a stenosis in the population will eventually die of CHD In the occurrence of the fatal cases individuals prone to an exceptionally progressive development of coronary artery disease play a considerable role This portion of the coronary deaths probably originates from a small fraction of the population

Although there was no difference in the areas of raised lesions between men born in the west and the east the men did differ with regard to the tendency to develop a coronary stenosis Within the upper half of the RL area range men born in the east were consistently shown to have a stenosis while the occurrence in men from the western counties was more random The ratio of the prevalence of a stenosis was 2 3 in the western and eastern men with an RL area of 4–12 cm² which is a similar ratio to that found in the CHD mortality rate between the western and eastern male populations (*Pyorala* 1974) The CHD mortality in men from these regions is probably closely related to the tendency to develop a coronary stenosis in association with an advancement of the coronary artery involvement due to RL This is supported by previous studies which have demonstrated more severe coronary calcification in men from eastern Finland (*Mottonen* 1969 *Rissanen* 1974) An increased tendency to calcification clearly increases the risk of a stenosis Present findings in men from the remaining counties are also in accordance with the hypothesis

CHD runs a short course and has a poor prognosis among men living in eastern Finland (*Punsar* 1972) A probable explanation is progressive development of coronary artery disease in many eastern CHD patients Severe stages of coronary artery involvement tended to be less frequent in SD patients born in the west Hence there are probably two background factors concerning the regional difference in the CHD mortality in Finland 1) the tendency to form a stenosis by the advancement of the RL extent is stronger and 2) the progressive development of coronary artery disease in CHD patients is more prevalent in the east than in the west It is possible that response of the coronary arteries to the risk factors is different in these populations Recent epidemiological studies have shown that correlation of hypercholesterolemia hypertension and smoking with a risk of CHD death is stronger in men living in eastern and central parts of Finland than in their western compatriots (*Pyorala et al* 1979)

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Acta path microbiol scand Sect. A 88 175–178 1980

IMMUNOHISTOCHEMICAL DEMONSTRATION OF A HITHERTO UNDESCRIBED LOCALIZATION OF HEMOGLOBIN A AND F IN ENDODERMAL CELLS OF NORMAL HUMAN YOLK SAC AND ENDODERMAL SINUS TUMOR

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Albrechtsen R Wewer U & Wimberley P D Immunohistochemical demonstration of a hitherto undescribed localization of hemoglobin A and F in endodermal cells of normal human yolk sac and endodermal sinus tumor Acta path microbiol scand Sect A 88 175–178 1980

In this study of 4 human yolk sacs the presence of hemoglobin A and F (HbA and HbF) is demonstrated for the first time in epithelial cells (type 1) and erythroid like cells (type 2) in the endodermal layer by immunoperoxidase technique Our findings strongly support the hypothesis previously proposed that the red blood cells formed in the yolk sac are of endodermal origin Tumor with yolk sac differentiation (8 endodermal sinus tumors and 1 embryonal carcinoma with vitelline areas) similarly showed HbA and HbF localisation in endodermal cells None of 59 germ cell tumors of other types contained these hemoglobins in the neoplastic cells

Key words Hemoglobin A and F human yolk sac endodermal sinus tumor immunohistochemistry

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The earliest erythropoiesis in human fetal life is generally accepted to arise from mesenchymal cells lying close to the yolk sac and allantois (9) However origin from the endoderm of the yolk sac has been suggested by several authors based on their findings of primitive erythroblasts in this layer (3 5 10) During investigations of the localization of a series of onchofetal antigens in the normal human yolk sac and in endodermal sinus (yolk sac) tumors we have observed the presence of focal endogenous peroxidase activity confined to both normal and neoplastic yolk sac cells The purpose of the present study was to investigate whether this peroxidase activity was due to the presence of hemoglobin

MATERIAL AND METHODS

The material includes 4 human yolk sacs obtained from operated tubal pregnancies and 68 cases of testicular and ovar[illegible] [illegible] according to Teilum s histogenetic classification (1965 [illegible] [illegible] used against AFP HbA and IgG from Dako Denmark and HbF (γ-chain) from Behringwerke Hoechst Denmark) In this method 10% H_2O_2 in methanol is used for total inhibition of endogenous

Fig 1 Wall of the yolk sac of a 6 week human embryo showing the presence of HbA in type 1 and 2 cells of the endodermal layer (EL) M indicates the mesenchyme (IP technique × 105)

Fig 2 Same case as in Fig 1 HbA demonstrated in typical endodermal cells (type 1) (IP technique × 730)

Fig 3 Same case as in Fig 1 HbA demonstrated in type 2 cells (erythroid like cells) of the endodermal layer Note the cell (X) with an epithelial like upper contour (as type 1) and a rounded lower margin (as type 2) (IP technique × 730)

Fig 4 HbF demonstrated in an endodermal sinus tumor Several of the endodermal cells (E) show positivity (IP technique × 180)

Fig 5 Embryonal carcinoma with vitelline areas HbF is seen in several of the tumor cells The insert shows a vessel close to the demonstrated area with erythroblasts (B) staining positive for HbF (IP technique × 204)

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peroxidase activity in the tissue sections The sections were counterstained with haematoxylin The visualisation of the peroxidase activity was made optimal with an interference filter fitted to the microscope which changed the reaction product from brown to red (4) Absorption of the specific antisera with corresponding antigens (AFP HbA and HbF from hemolysed adult and fetal blood) and using non immunized rabbit serum in place of the specific antisera served as negative controls

RESULTS

In all 4 yolk sacs variable intensity of AFP was localized only to the endodermal cells HbA and HbF were both demonstrated in all 4 yolk sacs in 2 different cell types of the endodermal layer Type 1 cells were columnar epithelial cells lying singly at some distance from each other and mainly localized to the middle and deeper portions of the endodermal layer (Fig 1 2) Type 2 cells were round erythroid like cells with same localization as type 1 (Fig 3) Transformation from type 1 to type 2 was often indicated as the contour of some cells on one side was characteristic for an epithelial cell (type 1) and round on the other side as an erythroid like cell (type 2) (Fig 3)

Clusters of mainly type 2 cells intermingled with a few type 1 cells were found predominantly in the part of the endodermal layer which spread to the mesenchyme having the appearance of blood islands Some mesenchymal cells including the pericytes also showed positive reaction but in all 4 cases this positivity was significantly less marked than in the endodermal cells Attempts at demonstration of IgG in the endodermal cells were constantly negative in all 4 cases

Demonstration of HbF (Fig 4) HbA and AFP in the tumors showed positive reaction only in areas with endodermal sinus tumor differentiation (8 out of 15 cases) and only in the endodermal cells We were not able to discriminate the characteristic type 1 and type 2 cells in the tumors as in the normal yolk sac Only one of the other germ cell tumors an embryonal carcinoma also showed positive reaction for HbF (Fig 5) HbA and AFP IgG could not be demonstrated in the tumor cells The remaining germ cell tumors which included seminomas embryonal carcinomas and mature teratomas were negative for HbF HbA and AFP in tumor cells in successive sections The specificity of the immunohistochemical staining technique for HbA and HbF was confirmed by the constantly positive reaction of the erythrocytes in the normal human fetus and tumors

COMMENTS

The present study shows for the first time the localization of HbA and HbF in the human yolk sac endoderm The absence of IgG in this layer excludes passive indiffusion of hemoglobin (1) Also phagocytosis of hemoglobin material by the endodermal cells is unlikely as the intracellular distribution was not localized focally but diffusely in each cell cytoplasm It may thus be concluded that the endodermal cells in the human yolk sac are able to synthesize hemoglobins thereby supporting the hypothesis previously proposed that the primitive erythroblasts formed in the yolk sac are of endodermal origin Furthermore we have demonstrated that HbA and HbF are present in human yolk sac as early as 6 weeks of age The expected presence of embryonic hemoglobins (6) could not be confirmed immunohistochemically in this study as no antisera are yet available However the isolation and purification of the embryonic hemoglobins (2 11) have recently opened the possibility of the production of such antisera

Of the germ cell tumors investigated it was only in those with endodermal sinus tumor differentiation that localization of HbA and HbF was found and confined to the endodermal cells as in the


Type I
Type II
M
EL
1
Type II
2
Type II
X
3
E
4
E
B
5

HEART PATHOLOGY IN RATS FED PARTIALLY HYDROGENATED FISH OIL, RAPESEED OIL OR PEANUT OIL FOR 30 WEEKS

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Svaar H & Langmark F T Heart pathology in rats fed partially hydrogenated fish oil rapeseed oil or peanut oil for 30 weeks Acta path microbiol scand Sect A 88 179–187 1980

132 male *Sprague Dawley* rats were given diets for 30 weeks including rapeseed oil with 41 4% erucic acid partially hydrogenated fish oil with 15 1% docosenoic acids or peanut oil with no docosenoic acids Four diets were isocaloric and contained respectively 21% rapeseed oil (8 7% w/w erucic acid) 10 5% rapeseed oil and 10 5% peanut oil (4 4% w/w erucic acid) 21% partially hydrogenated fish oil (3 2% w/w docosenoic acids) and 21% peanut oil The fifth diet contained 4 3% peanut oil The relative heart weights increased in rats fed rapeseed oil and partially hydrogenated fish oil and abnormally enlarged hearts were found in 32% of the rats fed 21% rapeseed oil and in 5% of those fed 10 5% rapeseed oil Heart lesions consisting of focal or confluent destruction of muscle cells were seen in all groups The incidence was 96% and the average severity grade 2 5 when 21% rapeseed oil was given and 61% and 1 3 respectively when 10 5% rapeseed oil was given Minor heart lesions were found in 14% of the rats fed 21% partially hydrogenated fish oil in 39% of those fed 21% peanut oil and in 12% when 4 3% peanut oil was given It is concluded that partially hydrogenated fish oil is markedly less cardiopathogenic than high erucic rapeseed oil The heart lesions that were found do not differ in incidence severity or morphology from those found when peanut oil was given or from those reported when other control fats and oils are given to rats for prolonged feeding periods

Key words Fish oil rapeseed oil heart lesions rats

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Long term feeding of rats with diets containing rapeseed oil rich in erucic acid (C22 1 cis omega 9) is accompanied by heart muscle destruction histiocytic infiltration and scar tissue formation (2 3 5 6 8 15) Male rats of the *Sprague Dawley* and *Wistar* strains are particularly sensitive to these diets (3 12 18) The erucic acid has been considered to be the main cardiotoxic factor since shortly after intake heart muscle lipidosis occurs to a degree that is directly related to the dietary concentration of erucic acid There is no known explanation for the heart muscle destruction however since the lipidosis is transient and the heart muscle lesions are seen after prolonged feeding periods (12)

The transient lipidosis phenomenon [illegible]

[illegible] only a small proportion is erucic acid while the balance is made up of other positional and geometrical isomers of erucic acid (13) We have previously examined the heart morphology in rats fed partially hydrogenated fish oils for 16 weeks and we were unable to find cardiac lesions that could be ascribed to these oils since similar changes of the same incidence and severity were found in the rats fed lard and corn oil mixtures as control fats (16)

The present study was therefore undertaken to observe the cardiac changes after prolonged feeding

normal human yolk sac This is expected from the finding that this tumor type is well characterized by a selective overgrowth of yolk sac endoderm and that hematopoiesis can often be found in this tumor in contrast to other germ cell tumors (13) *Kellen & Malkin* (1979) have shown significant elevation of HbF in the blood of some patients with ovarian and testicular cancers compared to other cases of malignant tumors Our results suggest that this finding could be due to the tumors own production of HbF Further studies are required to elucidate whether fetal and embryonic hemoglobins could be useful markers for endodermal sinus tumors

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The transient lipidosis phenomenon also occurs when partially hydrogenated fish oils are given (17) The latter oils contain 10–30% C22 1 fatty acids of which only a small proportion is erucic acid while the balance is made up of other positional and geometrical isomers of erucic acid (13) We have previously examined the heart morphology in rats fed partially hydrogenated fish oils for 16 weeks and we were unable to find cardiac lesions that could be ascribed to these oils since similar changes of the same incidence and severity were found in the rats fed lard and corn oil mixtures as control fats (16)

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TABLE 2 *Fatty Acid Composit on of D etary Oils* (w/w %)

Fatty acid	PO	PHFO	RSO
12 0	–	0 1	–
14 0	0 1	8 2	0 1
15 0	–	0 4	–
16 0	10 9	13 7	2 7
16 1	0 2	8 9	0 2
17 0	–	0 6	–
17 1	0 1	–	–
18 0	3 0	3 4	1 1
18 1	37 3	15 9	16 8
18 2	40 6	2 5	14 9
18 3	–	–	9 0
19 0	–	0 3	–
20 0	1 2	1 9	0 6
20 1	1 8	16 2	9 4
20 2	–	–	0 7
20 poly	–	5 0	–
21 0	–	0 5	
22 0	3 2	2 1	0 6
22 1	–	15 1	41 6
22 2	–	–	0 6
22 poly	–	4 1	–
24 0	1 6	–	0 2
24 1	–	1 1	–
Others	–	1 1	

Diets Five diets were prepared with the composition shown in Table 1 All the oils were commercial samples obtained from *DeNoFa/Lilleborg Fabr kker* Fredrikstad Norway The fatty acid composition of the oils used is shown in Table 2 The partially hydrogenated fish oil was produced from capelin oil and had a melting point of 31–33 °C The diet contained 3 2% w/w C22 1 and 3 4% w/w C20 1 The rapeseed oil diets contained 8 7% w/w and 4 4% w/w C22 1 (erucic acid) and 2 0% w/w and 1 1% w/w C20 1 respectively The peanut oil diets had no C22 1 fatty acids and 0 4% w/w C20 1 The low fat peanut o l diet had a fat amount sim lar to the commercial pellets used as standard rat food

RESULTS

All diets were well tolerated and the rats had a steady weight increase up to the 24th week At this time an increasing number of rats fed 21% rapeseed oil or 21% peanut oil began to eat less and to lose weight These rats appeared irritable but no other signs of sickness could be detected All the diets were analyzed to verify composition but no faults were detected The weight loss continued in the two groups even with newly prepared diets including fresh salt and vitamin mixtures The other three groups had a weight increase even after week 24 but their growth curves levelled off and from week 27 a weight loss was found also in these groups (Fig 1) The growth rate for the first 24 weeks was highest in the group fed 21% peanut oil and significantly lower in the group fed partially hydrogenated fish oil The lag in weight increase was most pronounced during the first 8 weeks afterwards the weight increase stabilized at a level of 71–76% of the peanut oil group

Five rats fed partially hydrogenated fish oil died in the early stages of the experiment (after 2 8 9 10 and 11 weeks) They had all appeared healthy and their weight increases were similar to the other rats in the group up to the time of death One of these rats had severe degeneration and inflamma tion of the epididymis and all of them had dilatation of the kidney tubules and calcification of some tubular cells The other organs had normal morphology at autopsy and no evidence of cardiac lesions was found One rat fed 10 5% rapeseed oil + 10 5% peanut oil appeared to be sick for one week A malignant tumor was then discovered and

nia was found at autopsy

Symptoms of essential fatty acid (EFA) – deficiency were sought with particular care but during the experimental period no signs of scaliness of the tails or feet were found in any of the rats no hairloss was observed and all the rats developed a brown yellow colour of the fur on their backs

At autopsy the relative organ weights in the rats fed 4 3% and 21% peanut oil were similar while the mean weights of the lung the heart and the kidney were increased in the rats fed 21% rapeseed oil and the heart the liver and the kidney were enlarged in the rats fed 21% partially hydrogenated fish oil ($p < 0.01$ *Wilcoxon* rank sum test) Micro scopical changes in the lungs the livers and the kidneys *are summarized in Table 3 Focal intersti* tial pneumonia was found in 13% of the rats Accumulation of lipid-containing foam cells in the lung tissue was more frequent in rats fed the fat rich diets Steatosis in the livers was moderate and involved individual liver cells Calcification of ind vidual kidney tubular cells was seen in all groups but most frequently in the rats fed [illegible]

[illegible] body weight and the hearts were considered to be abnormally enlarged when they weighed more than 0 43 g/100g body weight

of partially hydrogenated fish oil Two diets containing peanut oil were chosen as »negative« controls and two diets containing rapeseed oil rich in erucic acid served as »positive« controls

MATERIAL AND METHODS

Feeding 132 male *Sprague Dawley* rats were obtained from *Anticimex* Sollentuna Sweden when 3 weeks old They were fed commercial pellets for one week in individual cages before being randomly distributed to one of five experimental diets The rats were kept in one room at 21–22 °C at a relative humidity of 40–50% in wire mesh cages with elevated floor They had access to tap water *ad libitum* The diets were given in plastic cups at 10g per day the first 4 weeks 15g per day the next 4 weeks and 20g per day during the rest of the experiment Food consumption appeared to be similar in all the groups as judged from leftovers in the cups and spills in the cages but these amounts were not weighed The rats were handled once a week for weighing and inspection

Pathology The animals were killed with an overdose of ether A complete autopsy was performed and after weighing on a *Mettler* 2000C electric balance the organs were fixed in 4% neutral formaldehyde Ten hearts selected by random numbers from each group were examined for evidence of cardiac hypertrophy the right ventricle wall was carefully dissected from the interventricular septum and the left ventricle wall and both parts weighed separately All the hearts were divided in 5–6 transverse slices that were embedded in paraffin Two sections were made from each block One was stained with hematoxylin and eosin and the other with *van Gieson* for demonstration of connective tissue Sections from all organs were stained with hematoxylin and eosin and in addition the kidney sections were stained with *von Kossa* for demonstration of calcium salt

Five hearts selected at random from each group were perfused for 3–4 minutes with 2% glutaraldehyde in *Sørensen s* phosphate buffer via a canula in the aorta at a pressure of 75 cm water The hearts were cut in transverse sections and small pieces from the left and right papillary muscles and the left and right ventricle walls were postfixed in osmium tetroxide and embedded in Epon 812 Ultrathin sections were cut with glass knives on *LKB Ultratomes* stained with uranyl acetate and lead citrate and studied with a *Zeiss* EM9 electron microscope The remainder of the transverse heart sections were processed for light microscopy as above All the rats and the histological sections were examined without knowledge of the dietary groups until the study was completed

The heart lesions were scored according to the following grading system grade 1 = 1–3 small focal areas with muscle cell destruction grade 2 – more than 3 small lesions or 1–3 large lesions grade 3 – more than 3 large lesions grade 4 = transmural lesions A small lesion covers an area less than the microscope field at magnification 40 × 1 25 × 10 using a *Reichert binpus* microscope A large lesion covers an area larger than one field

TABLE 1 *Composition of the Diets in Grams pr 10 000 Grams*

	PO 4 3	PO 21	PHFO 21	RSO 10 5	RSO 21
Sucrose	2 000	2 000	2 000	2 000	2 000
Corn starch	4 820	3 150	3 150	3 150	3 150
Casein*	2 000	2 000	2 000	2 000	2 000
Cellulose	100	100	100	100	100
Salt mixture**	500	500	500	500	500
Vitamin mixture***	150	150	150	150	150
Peanut oil (PO)	430	2 100		1 050	–
Partially hydrogenated capelin oil (PHFO)	–	–	2 100	–	–
Rapeseed oil (RSO)	–	–	–	1 050	2 100
% C22 1	0	0	3 2	4 4	8 7

*Casein supplemented with 40 g methionin pr 2 000 g
**AOAC Methods XIth edition 1970 p 800
***AOAC Methods XIth edition 1970 p 885

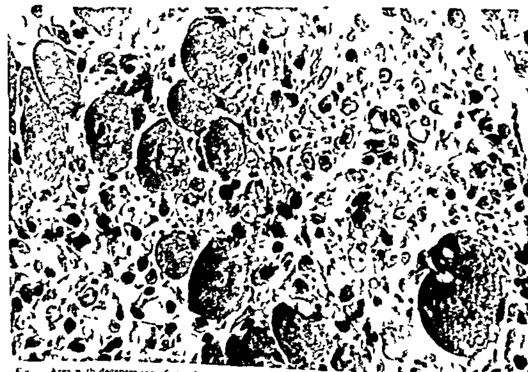
PO 4 3 = 4 3% w/w peanut oil in diet
...l in diet
...enated fish oil in diet
...nd 10 5% w/w peanut oil in diet
...et

TABLE 4 *D str but on of Heart Les ons in Groups*

Number of rats in group	PO 4 3 26	PO 21 26	PHFO 21 22	RSO 10 5 26	RSO 21 25
Macroscop cal les ons				27%	48%
M croscop cal lesions					
Grade 1	12%	27%	9%	19%	8%
Grade 2		8%	5%	19%	20%
Grade 3		4%	–	23%	68%
Hearts with les ons	12%	39%	14%	61%	96%
A erage grade per heart	0 1	0 5	0 2	1 3	2 5
% C22 1 n d et	0	0	3 2	4 4	8 7

(e heart weight in control groups + 4 standard deviations) Abnormally enlarged hearts were found in 32% of the rats fed 21% rapeseed oil and one rat fed 10 5% rapeseed o l had a heart weight of 0 63 The hearts were symmetrically enlarged and the ratio of the interventricular septum + left free wall we ght/right ventricle wall weight was 3 9 ± 0 4 in all groups

By macroscopical examination patchy fibrosis was found on the epicardial surface of 19 hearts all from rats fed rapeseed oil The distribution of the microscopical heart lesions is shown in Table 4 The lesions were in general located in the outer 1/3 of the left and right ventricle walls (the subep cardial zone) but in the rats fed rapeseed oil lesions were also found in other parts of the walls fairly



F g Area w th degenera ion of muscle cells necros s and nf ltration of h stiocytes rat fed RSO 21 500 ×

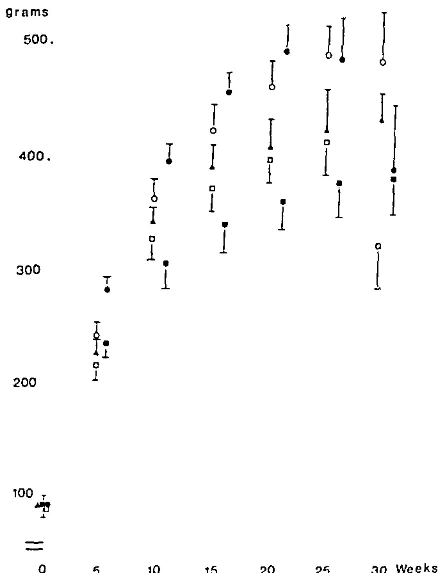


Fig 1 Average growth of male Sprague-Dawley rats fed the test diets for 30 weeks (with SD)
▲ 4 3 % peanut oil PO 4 3
● 21 % peanut oil PO 21
■ 21 % partially hydrogenated fish oil PHFO 21
□ 21 % rapeseed oil RSO 21
○ 10 5 % rapeseed oil + 10 5 % peanut oil RSO 10 5

TABLE 3 *Morphological Changes in Lungs Livers and Kidneys*

	PO 4 3	PO 21	PHFO 21	RSO 10 5	RSO 21
Number of rats in group	26	26	22	26	25
Lungs					
Pneumonia	15 %	12 %	0	12 %	20 %
Granuloma	4 %	12 %	23 %	15 %	16 %
Foam cells	12 %	58 %	41 %	50 %	64 %
Livers					
Steatosis	0	0	4 %	0	20 %
Kidneys					
Calcified tubules	12 %	4 %	50 %	15 %	20 %

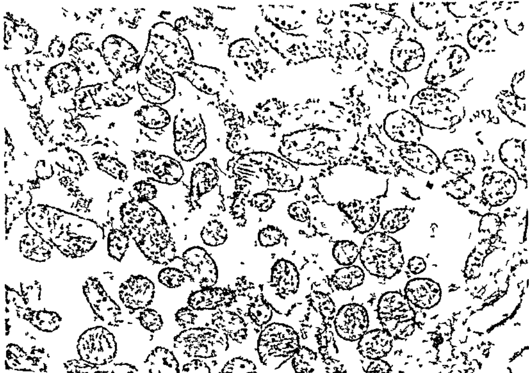


Fig 4 Increase of mitochondria and loss of myofibrils in heart muscle Same heart as fig 3 15 000 ×

96% when the rapeseed oil amount was increased to give 1 6% w/w erucic acid In these studies there seems to be a good correlation between the incidence and severity of the heart lesions and the amount of rapeseed oil in the diet but the erucic acid concentrations in the diets causing 50–60% incidence varies considerably (4 4% 1 1% and 0 6%)

There has been some speculation as to other factors in rapeseed oil that contribute to the cardiopathogenic effects Partial hydrogenation of rapeseed oil rich in erucic acid was accompanied by a reduction in the cardiopathogenic effects in male *Sprague Dawley* rats fed for 16 weeks (5) Partial hydrogenation reduced the content of linoleic acid and linolenic acid and increased the proportion of saturated fatty acids *McCutcheon et al* (14) fed semipurified diets with fat from different vegetable sources to male *Wistar* rats for 25 weeks The most severe heart lesions were found in rats given 5–6% w/w erucic acid irrespective of the vegetable source (two rapeseed oils *Brassica napus* and *Brassica campestris* and oil from nasturtium *Trapaeolum majus)* and the incidence was 100% Similar heart lesions were found in 50% of the rats fed a low-erucic rapeseed oil (*Brassica napus*) with 0 15% erucic acid while one diet containing 5 5% w/w erucic acid and no linolenic acid resulted in a marked reduction in the incidence/severity rating of the heart lesions The authors therefore postulated that linolenic acid plays a role in the cardiopathogenicity of rapeseed oil *Hulan et al* (10) obtained rendered pig fat containing 5 6% erucic acid after having fed rapeseed oil to pigs for 16 weeks When the pig fat was given to *Sprague Dawley* rats for 16 weeks minor heart lesions were found with the same incidence as in rats fed the control fat (lard) A similar incidence and severity was found when lard supplemented with 5 6% free erucic acid was given while *Span* rapeseed oil *(Brassica campestris)* containing 4 8% *erucic acid* caused a significant increase in the incidence and severity of the heart lesions These results suggest that erucic acid is not alone responsible for the heart lesions

In the present study peanut oil was given as control fat at two levels 4 3% and 21% Heart lesions with the same morphological appearance as found after rapeseed oil feeding were seen in both groups but more pronounced changes were found [illegible]

unexplained weight loss during the last 6 weeks of the experiment and this may be of importance Most

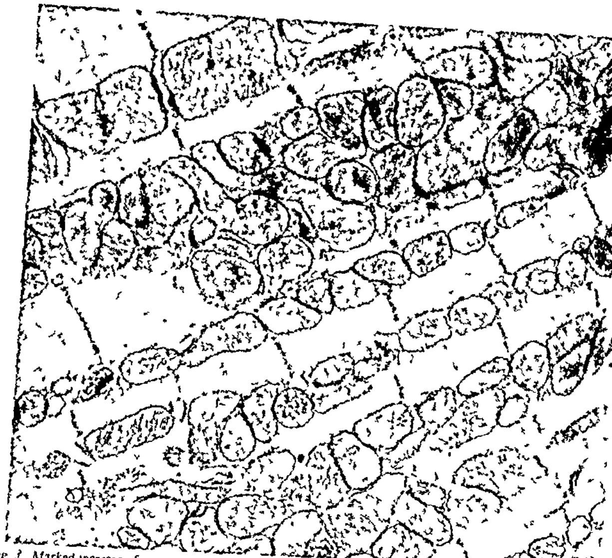


Fig 3 Marked increase of mitochondria in heart muscle from rat fed RSO 10 5 The heart was markedly enlarged 15 000 ×

frequently in the subendocardial zone in the left ventricle wall Degenerating or necrotic muscle cells were seen in all hearts scored as positive in addition infiltrations of histiocytes were found together with areas of fibrotic scar tissue (Fig 2) Electron microscopical examination of the largest heart showed a marked increase of mitochondria (Fig 3) and focal areas with loss of myofibrils in the heart muscle cells (Fig 4) The partially hydrogenated fish oil had produced minor lesions in 3 out of 22 hearts (14 %) and the changes appeared similar to those found in the other groups

DISCUSSION

In this study the pathological effect of rapeseed oil rich in erucic acid given to male *Sprague Dawley* rats is clearly demonstrated The main changes in the heart are muscle cell necrosis and scar tiss formation More pronounced changes appear whe the dietary concentration is increased from 50 % t 100 % of the fat (erucic acid content from 4 4 % w w to 8 7 % w/w) the incidence increased from 61 % to 96 % and the severity grade from 1 3 to 2 5 Th results confirm other studies with long term feed of this type of oil to rats Thus *Abdellatif & Vles* (3 found heart lesions in 50 % of male *Sprague Dawley* rats fed 25 % rapeseed oil (11 1 % w/w erucic acid) for 32 weeks and the incidence increased to 88–100 % when the rapeseed oi constituted 62 %–75 % of the dietary fat (5 5 %–6 5 % w/w erucic acid) They found similar changes when 30 % w/w rapeseed oil or 15 % w/w trierucin was given to *Wistar* rats for 24 weeks (each die contained 12 5 % w/w erucic acid) *Ziemlanski et al* (19) found an incidence of 61 % when a 5 % rapeseed oil diet with 0 6 % w/w erucic acid was given for 28

The excellent facilities and good help supplied by Dr med vet. L Scherven and the Animal Unit, Rikshospita et, Oslo are acknowledged The technical assistance of Mrs G Berg Pedersen is greatly appreciated DeNoFa og Lilleborg Fabrikker Fredrikstad supplied the oils and Mr T Grimsvang and Mr B Bauge were responsible for the fatty acid analysis This work was supported by the *Royal Norwegian Council for Scientific and Industrial Research* and the *Norwegian Research Association for Edible Fats*

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investigators studying the cardiac effects of fats and oils include reports on cardiac lesions in the experimental animals receiving the control diets The incidence varies considerably from experiment to experiment but the average incidence seems to be about 20% with a range from 0 to 40% *Kaunitz &*

experiment with male *Charles River* rats Unspecific degenerative changes consisting of focal muscle cell destruction histiocytic infiltrations and focal fibrosis were frequent The incidence increased with age from 26% of those dying between 400 and 500 days to 80% of those dying over 900 days of age The most severe degenerative changes were brought about by the relatively unsaturated vegetable oils (corn oil soyabean oil and olive oil) while the relatively more saturated animal fats (beef fat butter chicken fat and lard) induced fewer cardiac changes than expected after age correlated analysis

In this study the partially hydrogenated fish oil was given without supplement of essential fatty acids and this may be a serious objection to the experimental design The oil contained 2 3% C18 2 and this corresponds to 1 cal% of the diet a level that has been considered the minimal daily requirement for rats (9) But most likely only a fraction of C18 2 in this oil is the essential fatty acid C18 2omega6 (linoleic acid) Aaes Jørgensen (1) induced EFA deficiency after 7–8 weeks with partially hydrogenated fish oils free of EFA and this suggests that the fatty acid composition in such oils increases the requirement for EFA since 24–26 weeks on a fat free diet and 16–17 weeks on partially hydrogenated peanut oil were needed to induce similar evidence of EFA deficiency in the rats Unfortunately the most sensitive indicator of EFA deficiency the ratio between C20 3 and C20 4 in the serum was not determined in our study Other indicators of EFA deficiency such as weight curves appearance of the skin and tail histology of the testicles the kidneys and the livers have been closely examined The testicles were normal with mature spermatozoa in the tubules The kidney weight was increased (+ 22 % of controls) and focal calcification of tubular cells was found in 50% of the rats The liver weight was increased (+ 37% of controls) and 4% had moderate steatosis Thus some findings may indicate that the rats fed partially hydrogenated fish oil were deficient in EFA but these indicators are not specific since similar changes were found in other groups particularly in the group fed 21% rapeseed oil (Table 3)

The groups fed 21% partially hydrogenated fish oil and 21% rapeseed oil had very similar weight increases during the first 4 weeks the next 4 weeks the weight increase was 10% less in the group fed partially hydrogenated fish oil and in the following 16 weeks the two groups developed at the same rate. This suggests that the absorption rates were fairly similar for the two diets since no difference were observed in the food consumption Most likely the rats fed partially hydrogenated fish oil had borderline EFA deficiency but corn starch as used in all the diets probably contained enough essential fatty acids to prevent manifest symptoms EFA-deficiency appears to affect the synthesis or release of chylomicrons while the intestinal absorption appears unchanged (7) To our knowledge there are no data to indicate that the dietary essential fatty acids influence the utilization of C22 1 fatty acids to a higher degree than other fatty acids We therefore think it unlikely that the low incidence and severity of the cardiac lesions found in rats fed partially hydrogenated fish oils are due to a reduced utilization of C22 1 fatty acids The results of the histopathological examination of the hearts are in agreement with those found after giving similar partially hydrogenated fish oils to male *Charles River* and *Sprague Dawley* rats for 16 weeks (4 16)

Judging from long term feeding experiments with partially hydrogenated fish oil one can not exclude the possibility that their content of C22 1 fatty acids may have cardiopathogenic effects similar to rapeseed oils containing erucic acid Some recent observations from the studies cited above point to features of partially hydrogenated fish oil that may modify possible cardiac effects in the rats in a favourable direction they originate from an animal source and the oils contain more saturated fatty acids than most vegetable oils they have no linolenic acid content and the C22 1 fatty acids consist of a variety of geometrical and positional isomers of which erucic acid constitutes very little and trans isomers at least 50% (13)

At the present time it is not possible to know whether and to what degree these features are important in determining any pathological effects of partially hydrogenated fish oils Our experiment suggests that diets including partially hydrogenated fish oil containing 3% C22 1 fatty acids do not cause cardiac lesions to a greater extent than do vegetable oils commonly accepted as healthful or mixtures of fats and oils of animal and vegetable origin The few lesions observed are probably unspecific or related to the conditions of the experimental situation and a fat rich diet may be one of the conditioning factors

The excellent facilities and good help supplied by Dr med vet L Scheven and the Animal Unit, Rikshospitalet, Oslo are acknowledged The technical assistance of Mrs G Berg Pedersen is greatly appreciated DeNoFa og Lilleborg Fabrikker Fredrikstad supplied the oils and Mr T Grimsvang and Mr B Bauge were responsible for the fatty acid analysis This work was supported by the *Royal Norwegian Council for Scientific and Industrial Research* and the *Norwegian Research Association for Edible Fats*

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Acta path microbiol scand Sect A 88 189-194 1980

AN ESTABLISHED HUMAN CELL LINE DERIVED FROM A MALIGNANT MEDIASTINAL TERATOMA[1]

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Sundstrom C Mark J & Westermark B An established human cell line derived from a malignant mediastinal teratoma Acta path microbiol scand Sect A 88 189 194 1980

An established cell line derived from a human malignant mediastinal teratoma is described The cell l ne was c
exponentia
tissues we
Chromosome banding showed a hypertriploid stemline number S−78 Almost all of the analysed karyotypes had seven markers in common Eleven other markers occurring at low frequency were also detected The orig n of all markers except one could be deduced

Key words Teratoma malignant established cell line

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Accepted as submitted 17 xii 79

Numerous efforts have been made to establish permanent cell lines from human malignancies With a few except ons

of establishment is very low and only in the order of a few per cent (5) We have recently managed to establish a cell line from a malignant teratoma The present communication describes some of the features of this cell line designated U 1161

MATERIALS AND METHODS

Case report The patient was a 26 years old Caucasian male who had suffered from thoracical pains for four months prior to operation At surgery a mediastinal grapefruit-sized tumor weighing about 1000 g was removed The histological picture revealed a tumor with major parts consisting of mesenchymal fibrous tissue

of the tumor seminomatous areas were seen partly intermingled with a lymphoid component Within the mesenchymal and epithelial components a strong atypia was observed No thymic tissue could be found The histological appearance of the tumor was consistent with malignant teratoma partly with admixture of seminoma Embryonal carcinoma or teratocarcinoma was not present (7) Renewed histological examination of a pleural metastasis 3 months after the primary operation showed that all components of the original tumor were present although the atypia within the fibrous tissue part was more pronounced and the growth here was more sarcomatous

The tumor mass could not be radically excised and the patient died eight months after operation No autopsy was performed

Cell culture Pieces of fresh tumor obtained at primary surgery were immediately transposted to the laboratory and

containing 5% CO_2 Medium was changed twice weekly Dense cultures were subcultivated 1 2 after a brief treatment with 0 25% trypsin solution



Fig 2 Micrographs of cultures of cell line U 1161 (a) Low power view showing fibroblastic appearance of cultures with cells often lying in bundles (b) High power view showing nuclear shape of individual tumor cells

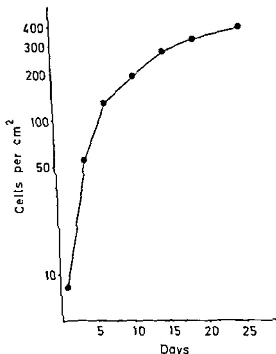


Fig 3 Growth curve of U 1161 Population doubling time during exponential growth was 24 h

Characteristics of the established cell line The cell line was uniformly composed of elongated spindle shaped cells with a notable fibroblastic appearance (Fig 2a) Most of the nuclei were oval but more irregularly shaped nuclei were commonly seen (Fig 2b) The chromatin pattern was coarse Most nuclei contained 1–3 heavily stained nucleoli A fraction of multinucleated cells was always present The growth pattern was also fibroblastic with cells growing in bundles (Fig 2a) although the growth pattern was more irregular than is usually seen in cultures of normal fibroblasts

Sparsely seeded cells multiplied rather rapidly and the population-doubling time during the phase of exponential growth was 24 h (cf Fig 3) As the cultures became denser growth rate progressively decreased until it became very slow at a cell density of 350 000 cells/cm^2 At this stage the cultures were very crowded and piling up of cells could be observed

The cultures could be subcultivated twice a week The cell line has now been grown for more than 100 passages

Table 1 shows the chromosome counts in the 155

TABLE 1 *Chromosome Counts in U 1161*

Chromosome numbers																							T
50–60	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	94	±160	
3	1	1	–	7^{3}	4^{2}	5^{2}	11^{4}	19^{5}	41^{10}	23^{7}	9^{3}	2	3^{1}	1	1	2	3	2	5^{1}	1	1	10^{2}	1

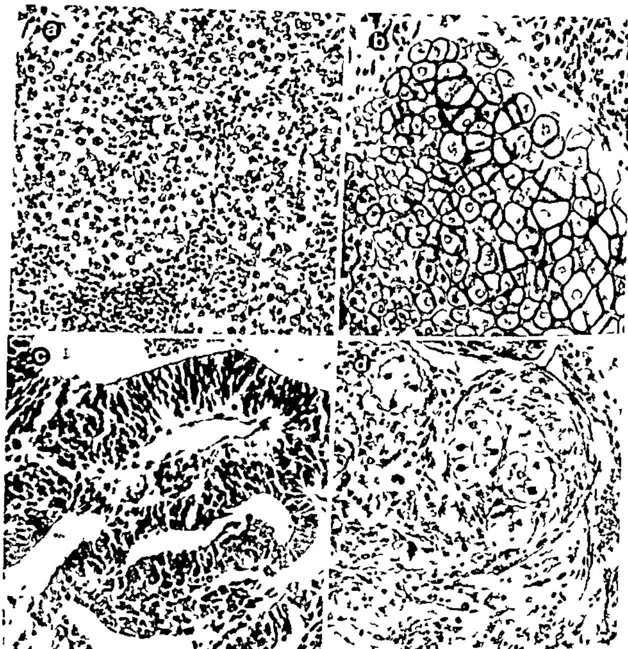


Fig 1 Micrographs show ng d fferent parts of the malignant teratoma from which cell line U 1161 was derived The tumor contained a mixture of different tissue elements for example as shown here parts with sem noma l ke appearance contain ng anaplastic tumor cells and surrounded by a stroma with lymphocytic inf ltrate (a) w th cartilage (b) with cystic spaces lined by irregular palisaded neural epithelium (c) and areas built up of irregular connective tissue conta n ng notochordal cells (d) In mesenchymal and epithelial components atyp a was noted (× 260)

Cell counts Cells were detached with trypsin and the cell number was determined on duplicates by the aid of an electronic particle counter (AB Lars Ljungberg Stock holm Sweden)

Cytogenet cal methods The chromosomes were studied in preparations of 4 different subcultures viz passages 34 37 70 and 75 The cytogenetical findings were similar in the different passages and therefore the results are combined below The methods for the chromosome preparations the G and C banding techniques and the nomenclature have been described in detail earl er *(3)*

RESULTS

Early cell culture characteristics The early out growth of cells was composed of a mixture of different cell types mainly epithelial cells fibro blast like cells and some large polygonal cells The mitotic activity of the epithelial and polygonal elements was low whereas the fibroblast like cells proliferated more rapidly leading to an increase in the proportion of these cells After approximately 20 subcultivations only fibroblast like cells were detected

TABLE 2 *The Representation of Normal Chromosome Types in 41 Karyotyped Modal Near-Modal Cells*

Chromosome No	Number of each chr type						The predominating number of each chromosome type
	0	1	2	3	4	5	
1	–	32	4	3	2	–	1
2	–	–	6	35	–	–	3
3	–	3	33	4	1	–	2
4	–	–	7	29	5	–	3
5	–	–	4	5	32	–	4
6	–	–	–	7	34	–	4
7	–	–	2	36	3	–	3
8	–	–	–	11	29	1	4
9	–	–	38	2	1	–	2
10	–	–	6	35	–	–	3
11	–	–	–	18	23	–	3–4
12	–	–	3	37	1	–	3
13	–	34	7	–	–	–	1
14	–	–	–	–	39	2	3
15	–	1	11	29	–	–	4
16	–	–	–	39	1	1	3
17	–	–	2	39	–	–	3
18	–	–	1	3	35	2	4
19	–	–	2	28	11	–	3
20	–	–	2	4	35	–	4
21	–	3	4	23	11	–	3
22	–	–	–	14	27	–	4
Y	2	6	33	–	–	–	2
X	–	4	37	–	–	–	2

TABLE 3 *The Derivation and Frequency of the Marker Chromosome in 41 Karyotyped Modal-Near Modal Cells*

Marker no	Number of cells with the marker	Mode of origin
I = 1q–	36	t(1 10)(1pter – 1q21 10q11 – 10qter)
II = 1q–	33	i(1q)
III = 1q–	36	t(1p 7q)
IV = 3q–	36	del (3)(q11)
V = 3p–	34	t(3q 7p)
VI = 10q +	34	t(1 10)(1qter – 1q21 10q11 – 10pter)
VII = 13p +	35	t(9q 13q)
VIII = 1p +	5	t(1 ?)(1qter – 1p36 ?)
IX = 1q–	4	del (1)(q11)
X = 2q +	1	t(2 ?)(2pter – 2q37 ?)
XI = 3q +	1	t(3 ?)(3pter – 3q29 ?)
XII = 5p +	1	t(5 ?)(5qter – 5p15 ?)
XII = 10q +	1	t(10 ?)(10pter – 10q26 ?)
XIV = 11p–	5	del (11)(p12)
XV = 12q–	1	del (12)(q23)
XVI = 21p +	1	i(21q)
XVII = Xq +	1	t(X ?)(Xpter – Xq28 ?)
XVIII = minute	2	very small biarmed marker with unknown derivation

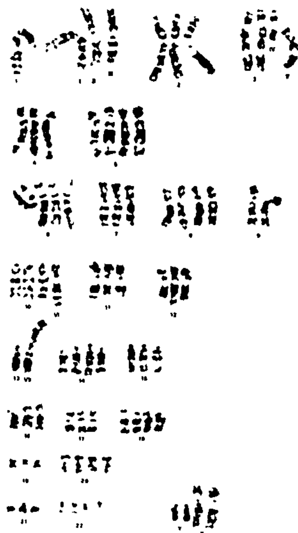


Fig 4 Karyotype of U 1161 showing a hypertriploid stemline (S = 78)

cells studied from the four different passages The distribution of the 41 karyotyped cells on the various chromosome numbers is indicated by the upper figures in the Table

The established cell line U 1161 had a hypertriploid stemline (S) number S = 78 There was a considerable spread around the modal number and a fairly low frequnecy of doubling products of modal-near-modal cells

Five of the analysed cells with the S number had the same karyotype This is illustrated in Fig 4, and it can be written as follows 78, XXYY 1 +2, +4, +5, +5, +6, +6, +7, +8, +8 +10 +11 +12, 13, +14, +14, +15, +16, +17, +18 +18 +19, +20, +20, +21, +22, +22, +7 markers (= mar I–VII) The origin of all the seven different marker types could be completely elucidated (Table 3) The results by C-banding corroborated the interpretations presented in Table 3, and the observations by this technique were especially valuable for characterizing the mar I, II, III and VII A striking feature as regards the formation of mar I–VII was the preferential involvement of the centromeric and near-centromeric regions

The 34 analysed variant cells with chromosome numbers in the modal region were all closely related to the S-cells This feature is very clear from the data given in Tables 2 and 3 In these 34 variant cells 11 new marker types were observed (mar VIII–XVIII, Table 3) Their derivation could be wholly or partly deduced, except for mar XVIII

Two octaploid cells were analysed The representation of normal chromosome types and markers accorded well with their origin from doubling products of cells of the modal group No new marker types were seen in these high polyploid cells

DISCUSSION

Teratomas are characterized by their content of all three germ layers They are most often benign tumors but occasionally malignant variants appear When occurring, malignancies are most often adenocarcinomas although other types of malignant tumors have been described (for a recent review, see ref no 1) In the present mediastinal tumor both the epithelial and mesenchymal components showed atypia However, the recurring tumor was more sarcomatous Also, the cell line that was established from the primary tumor specimen was mesenchymal with cells resembling normal fibrobalsts except for a certain cellular atypia and a somewhat more irregular growth pattern than is usually seen in normal fibroblast cultures The possibility that the cell line was composed of normal fibroblasts could be excluded by the fact that it has been subcultivated for over 100 passages Also the markedly abnormal karyotype is consistent only with neoplasia

The original tumor contained mesenchymal elements of different lineages Of all cell types fibroblasts are those most prone to grow in tissue culture It is conceivable that this is the reason why cells committed to the »fibroblastic lineage« overgrew the other cell types that were present in the early cultures Thus, the emerging permanent cell line although derived from a teratoma, was probably a differentiated one and not composed of pluripotent, uncommited cells In this respect U-1161 differs from mouse teratocarcinoma lines which are either pluripotent or undifferentiated nullipotent (4) [illegible] all our efforts to induce differen[illegible]
unsuccessful

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SOMATOPETAL TRANSPORT OF HORSERADISH PEROXIDASE FOLLOWING INCORPORATION INTO AXONAL SPROUTS AND AXONS IN NEUROMAS AFTER NERVE TRANSECTION

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Olsson T Somatopetal transport of horseradish peroxidase following incorporation into axonal sprouts and axons in neuromas after nerve transection Acta path microbiol scand Sect A 88 195–199 1980

Horseradish peroxidase (HRP) applied to the proximal stump of the facial nerve of the mouse 5 h after section diffused into the endoneurium and was incorporated by endocytosis into axons densely packed with organelles By 24 h to 16 days after the section uptake of the tracer into profiles of outgrowing axonal sprouts rich in vesicles and vacuoles was registered Subsequently HRP accumulated in correspond ng perikarya Tracer transport from neuromas was demonstrated 32 and 64 days after section In this way influences from outgrowing nerve sprouts and axons in neuromas may reach nerve cell bodies

Key words Horseradish peroxidase axons nerve transection

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It has been shown that somatopetal transport of macromolecules to nerve cell bodies occurs after leakage into damaged axons immediately after an axotomy (16) and later during reinnervation of the target organ after incorporation into nerve terminals (8 15 21) The biological significance of this phenomenon is unclear but it may be of importance for the nerve cell body s interaction with its periphery after axon injury (14) for further evaluation of these phenomena the present study was undertaken to obtain information about axonal uptake and somatopetal transport of macromolecules also during the period after axotomy when the axolemma is reconstituted and outgrowth of regenerating axonal sprouts starts In addition transport of macromolecules from axons in neuroma formation developed after impeded reinnervation was studied In order to obtain outgrowing sprouts in restricted areas which are easy to localize axotomy was performed by transection in the present study

MATERIALS AND METHODS

The left facial nerve of 60 to 70-day-old male Swiss albino mice was transected below its exit from the stylomastoid foramen and a strip of parafilm placed around the proximal nerve stump In groups of mice 2 mg HRP (Type II Sigma St Louis) was applied on the proximal nerve stump various time intervals after the transection (number of mice and time intervals see Table I) The mice were then perfused after 30 min with 2 5% glutaraldehyde in Sorensen s phosphate buffer pH 7 4 with 0 01% $CaCl_2$ added The distal parts of the [illegible]

[illegible] and time intervals see Table I) For estimation of the HRP accumulation in these neurons the contralateral ones were used as reference by section of the right nerve followed by immediate application of 2 mg of the tracer After 9 or 24 h the

Chromosome banding data on human teratomas are scanty *Sonta et al* (8) have published data on three testicular tumors viz one hyperdiploid (S = 53) seminoma one hypotriploid (S = 67) teratoma and one metastatic diploid (S = 46) embryonal carcinoma It is surprising that no marker chromosomes were included in any of these S = karyotypes In the present cell line the S = karyotypes contained seven markers the exact origin of which could be deduced

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For studies of HRP transport to neuronal perikarya 2 mg of the tracer was applied on the left proximal nerve stump in groups of mice various time intervals after section (number of mice and time intervals see Table 1) For estimation of the HRP accumulation in these neurons the contralateral ones were used as reference by section of the right nerve followed by immediate application of 2 mg of the tracer After 9 or 24 h the

TABLE 1 *Survey of the Material in Which HRP Was Applied on Proximal Stumps of Facial Nerves at Various Time Intervals after Transection Subsequently Incorporation of HRP into Axons and Accumulation of the Tracer in Perikarya Was Studied*

Time intervals		1 h	5 h	24 h	48 h	5 days	16 days	32 days	64 days
No mice for studies of	HRP uptake in axons	2	2	4	2	3	2	2	2
	HRP accumulation in perikarya	7	6	4	3	4	3	2	3

mice were perfused as described above the brain stems dissected out and postfixed for 30 min Frozen section 40 µm thick were cut and reacted for HRP (18) The intensity of the HRP reaction product in the left facial nucleus was then evaluated with a scoring procedure *(21)*

The morphological response of facial nerve cell bodies to a nerve transection was also studied Groups of 2–3 mice were perfused with 4% formalin 6 8 12 18 20 24 32 34 and 38 days after the axotomy and paraffin embedded sections stained with cresyl violet acetic acid

RESULTS

1 5 h after the section the distal parts of the axons in the proximal nerve stump showed a finely granular axoplasm HRP had diffused into the endoneurium and in between the axolemma and the myelin sheet but only occasionally intra axonal HRP labelling occurred By 5 5 h distal parts of many axons were densely packed with vesicles occasional dense core vesicles vacuoles and mitochondria HRP outlined these axons beneath the myelin sheet and many vesicles and vacuoles contained HRP (Fig 1) Clusters of outgrowing nerve sprouts had appeared by 24 5 h A basal lamina surrounded the clusters of sprouts which were partly embedded in a sparse Schwann cell cytoplasm In many cases sprouts were also seen associated with a Schwann cell belonging to a myelinated axon (Fig 2) Sprouts appearing in these two types of clusters hav previously been shown to represent collateral and, or terminal sprouts of single myelinated axons (19; Organelles such as vesicles vacuoles elongate tubular structures multivesicular bodies mitochon dria and dense lamellar bodies were seen in some o the nerve sprouts profiles In these many HR labelled vesicles and vacuoles occurred (Figs 3 an 4) The majority of HRP labelled and non labelle vesicles had a size between 40 and 80 µm bu vacuoles up to 260 µm were also registered Othe profiles of sprouts showed very few vesicles an vacuoles and HRP labelling was infrequent in thes areas Clusters of sprouts with a similar morpho logy were seen after 48 h and 5 days In mice use after 16 days each section showed fewer profiles of sprouts with large amounts of vesicles and vacuoles Such structures and intra axonal HRP labelling occurred only occasionally by 32 and 64 days At these time intervals neuromas built up of myelina ted and unmyelinated axons had developed (Fig 5)

No or only a faint blue black reaction product appeared in left sided perikarya when the tracer was applied 1 h after section whereas most nerve cell bodies showed a weak reaction product in mice used after 5 h The left sided perikarya contained a strong clearly visible HRP reaction product at all later time intervals and neurons used 48 h after section appeared to possess more reaction product than the right sided ones (Fig 6)

Fig 1 Axon densely packed with organelles 5 5 h after transection Note black HRP reaction product outlining the axolemma and HRP labelling of intra axonal vesicles and vacuoles × 11 000

Fig 2 Clusters of nerve sprouts 24 5 h after transection In the cluster to the right the sprouts are embedded in a Schwann cell belonging to a myelinated axon Note black HRP reaction product in the endoneurium × 11 000

Fig 3 Profile of sprout from a cluster similar to the one to the left in Fig 2 24 5 h after transection The profile contains vesicles dense core vesicles and vacuoles HRP reaction product outlines the sprout and occurs in membrane limited organelles × 26 400

Fig 4 Profile of sprout embedded in a Schwann cell belonging to a myelinated axon Note HRP reaction product in membrane limited organelles × 49 600



2

3

4



Fig 5 Myelinated and unmyelinated axons in a neuroma 64 days after section Note HRP reaction product in the endoneurium surrounding unmyelinated axons × 4 800



Fig 6 Granular HRP reaction product in facial nerve cell bodies in a mouse where HRP was applied 48 h after transection × 490

The morphological response of the facial nerve cell bodies consisted of dispersion of the Nissl substance an increase in cytoplasmic basophilia in the size of cells and nucleoli This was followed by a decrease in basophilia and still later a loss of neurons was registered These results are in accordance with those by *Torvik and Skjorten* (26)

DISCUSSION

Previously it has been shown that HRP leaks through injured axolemma and distributes diffusely in axons immediately after an axotomy (16) The present study showed that 1 h after axotomy HRP outlined an intact axolemma with no or only a slight axonal HRP uptake and transfer to perikarya Later 5 h after axotomy distal parts of axons in the proximal nerve stump were densely packed with organelles At this time intra axonal vesicles and vacuoles of endocytotic origin were formed Other vesicles and vacuoles in these axons may represent lysosomes (13) or budded parts of smooth endoplas mic reticulum (6)

By 24 h after axotomy profiles of axonal sprou containing vesicles and vacuoles were observe Such structures have been interpreted as growin tips of sprouts (2 10 11 17) The presentl observed endocytosis in *in vivo* sprouts shows th they are in this respect similar to growth cones *i vitro* whose endocytotic activity is well known (1 4 22 25) However they differ from growth cones in the developing cerebellum of the rat which ar *organelle poor and have a low uptake of HRP (5)* An increase in HRP reaction product in perikarya similar to that observed in the present study in mic used after 48 h has previously been observed in th chick visual system 6 75 to 18 h after axon injury (12) It may reflect an increased peripheral uptake of the tracer which can be due to larger amounts of nerve sprouts and/or higher endocytotic activity in individual sprouts Not only exogenous tracers but also endogenous proteins and glycoproteins are retrogradely transported in higher amounts out growth of axons after an axotomy (9) After somatopetal transport to perikarya HRP is localised to lysosomes (3) Nevertheless macromolecules may after endocytosis and intracellular transport affect the metabolism of the cell (7 23) It is therefore conceivable that the mechanism of incor poration and subsequent somatopetal axonal trans port of macromolecules during outgrowth of axons may be important for the understanding of how the nerve cell body s metabolism may be influenced by the periphery as proposed by *Griffin and Price* (11)

Tracer transport from the neuromas was obser ved in the present study indicating a pathway for introduction of substances which could reduce the neuron s regenerative capacity as proposed by *Spen cer* (24) As discussed above HRP uptake occur most extensively in axonal structures which are rich in vesicles and vacuoles In accordance with a previous study (20) such structures were observed infrequently at late times after nerve section The discrepancy between the conspicious accumulation of tracer in perikarya and the infrequent observation of HRP uptake by axons in the neuromas may be explained by a difficulty to localize the vesicle rich axonal structure which may be spread over larger areas caused by an indefinite growth of axons after nerve section (24)

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A NEW HYPOTHESIS FOR ALLOXAN DIABETES

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Boquist L A new hypothesis for alloxan diabetes Acta path microbiol scand Sect A *88* 201–209 1980

A new hypothesis (»Pi pH hypothesis) for alloxan diabetes is presented It is based upon data from our own studies and from the literature The following data and interpretations are assumed to be of special importance for the B-cytotoxicity of alloxan Inhibition of a mitochondrial sulfhydryl dependent transport system for inorganic phosphate (Pi) leading to increased concentration of Pi and decreased pH in the cytosol and to inhibition of NAD-dependent oxidations and oxidative phosphorylation mitochondrial lesion because of altered localization and concentration of Pi inhibited synthesis and glucose induced release of insulin at least partly due to a fall in intracellular pH and finally necrosis because of absent mitochondrial function An inverse relationship between Pi and pH may exist in the B-cells alloxan sensitivity being associated with high Pi and low pH Alloxan antagonism may be due to induction of low Pi and high pH in the cytosol The selectivity of the B cell for alloxan is believed to be associated with its free permeability for glucose

Key words Alloxan diabetes alloxan antagonism pathogenesis inorganic phosphate intracellular pH mitochondrial lesion

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Although the diabetogenic action of alloxan has been known for more than 35 years and in spite of intense research during this period the pathogenesis of alloxan diabetes is still unknown Several hypotheses for the development of this kind of experimental diabetes have been presented but none has so far been proved Since data from our own studies in this field have not been compatible with any of these hypotheses we have tried to find a new hypothesis that agrees with our own results and with data from the literature As preliminarily reported (*Boquist* 1978 a d and e *Boquist & Hagström* 1979 *Lorentzon & Boquist* 1979) this search has resulted in a working hypothesis tentatively called the Pi pH hypothesis which here is presented in more details with the addition of some new data.

Mitochondrial Lesion and Inorganic Phosphate

Selective necrosis of the pancreatic islet B-cells represents the ultimate effect of alloxan B-cytotoxicity and forms the basis of manifest diabetes Hence it is obvious that alterations in these cells play a central role in the development of this kind of diabetes Since it is generally considered that alloxan is toxic for the B-cells only during a short period of time probably within the first five to ten minutes after its administration into the circulation the earliest structural and biochemical changes in the B cells are most important for the study of the pathogenesis of alloxan diabetes Alterations recorded in these cells when hyperglycemia is manifest may well be of [illegible]

[illegible] been found to be localized to the mitochondria and the pattern of pyroantimonate precipitation (*Boquist* 1977 a) The mitochondrial changes have been verified by ultrastructural stereological methods (*Lorentzon & Boquist* 1979) The observations were started 10 min following alloxan administration and already at this time many mitochondria

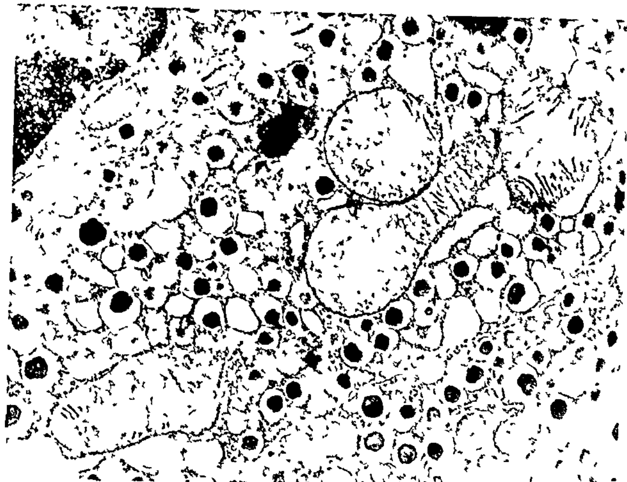


Fig 1 Electron micrograph of mouse B-cells 10 min after alloxan injection Swollen mitochondria with partly disrupted cristae are seen × 11 000

exhibited swelling and occasionally rupture of inner membranes (Fig 1) At the following observation times the mitochondrial changes were more advanced and subsequently the mitochondria were disintegrated

Since mitochondrial changes of the kind observed in the B cells were not found in the other endocrine cells or in the exocrine cells of the pancreas these changes obviously do not represent an artifact or unspecific reaction but seem to be a structural manifestation of a rapidly appearing specific lesion directly or indirectly caused by alloxan

The other alteration observed already at the 10 min observation time is a pattern of pyroantimonate precipitation in the B-cells of alloxan treated mice different from that of control mice Thus animals injected with alloxan exhibit precipitates mainly localized to the cytoplasmic ground substance (Fig 2) whereas most precipitates are seen in mitochondria and secretory granules in the controls (*Boquist* 1977 a) This may be consistent with an increased occurrence of Ca^{2+} and inorganic phosphate (P_i) *in the cytosol* and the finding thus supports the P_i pH hypothesis (see below)

The endoplasmic reticulum Golgi complex and nucleus are structurally altered first at late observation times and the secretory granules are usually preserved even in severely damaged but non necrotic B-cells (Fig 3) indicating an inhibition of the release of insulin stored in granules in alloxan treated mice This agrees with the observation that alloxan inhibits glucose induced insulin secretion (*Gunnarsson & Hellerström* 1973 *Henquin et al* 1979) This inhibition is abolished by glucose *in vitro* (*Tomita & Lacy* 1972) Alloxan also blocks insulin synthesis in isolated mouse islets (*Gunnarsson* 1975) Both qualitative and quantitative electron microscopic data support the view of an inhibition of the synthesis and release of insulin in alloxan treated mice (*Boquist* 1977a *Boquist & Lorentzon* 1979)

What is the mechanism underlying the mitochondrial lesion in alloxan treated mice? So far it has not been clarified whether alloxan passes the plasma membrane of the B-cell or not Therefore one possibility is that alloxan passes into the B-cell and acts directly on the mitochondria Another possibility is that alloxan through an action on

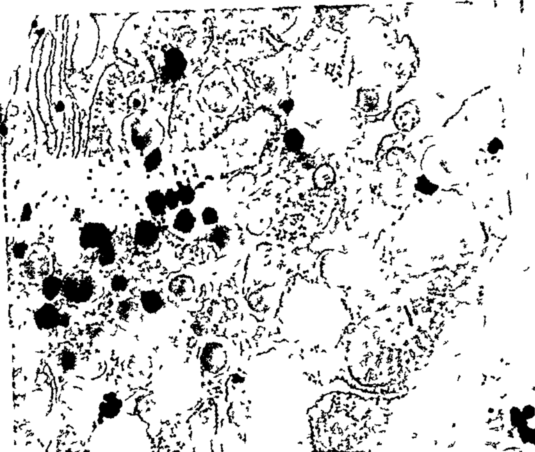


Fig 2 Portion of mouse B-cell 10 min after alloxan adm n ... te precipitates are present n the ... Some precipitate also found a ... Pyroantimonate techn que

other organelles or cellular constituents or through a direct action on the plasma membrane alters the intracellular m l eu in such a way that the mitochondria are secondarily damaged

The data from our own studies do not permit any definite interpretation of whether alloxan passes the B-cell membrane or not However the absence of early-appearing structural *changes* in organelles other than mitochondria argues against the possibi l ty of an action of alloxan on such organelles and although many authors advocate the

... d out damaging the plasma membrane (*McDaniel et al* 1975 *Weaver et al* 1978) or for an intracellular action besides plasma membrane effects (*Henquin et al* 1979) Autora d ography has revealed that *mouse islets* accumulate alloxan (*Hammarstrom et al* 1967) Moreover *in vitro* experiments have disclosed an action of alloxan on mitochondria from rat liver and kidney (*Younathan* 1962) Therefore it is believed that alloxan passes the plasma membrane of the B-cells supposedly through a hexose transport system and acts intracellularly In addition alloxan may directly or indirectly act on the plasma membrane

If alloxan really passes through a hexose trans port system the entry into the B-cells may not only be associated with that of glucose but also with that of sodium and Pi since glucose *and* Pi entry e g in the proximal tubule cells (which also are alloxan sensitive) of rabbit kidney is sodium-dependent (*Denn s & Brazy* 1978) Therefore the transport of

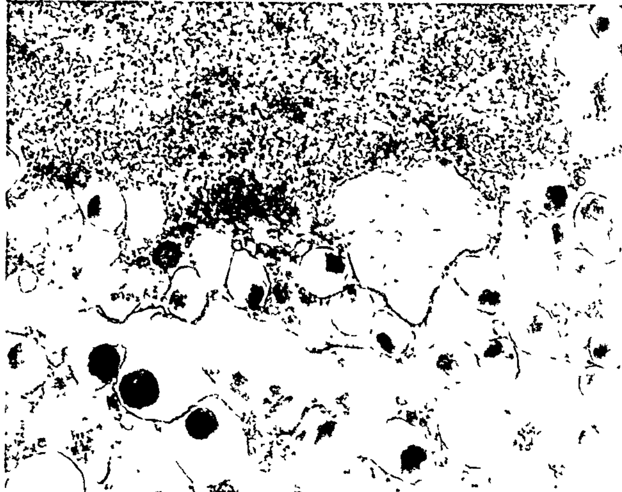


Fig 3 Portion of severely damaged B cell 6 hours after alloxan injection Note that some of the secretory granules exhibit an altered configuration of the core and/or rupture of the membrane × 19 000

sodium and Pi into the B-cells may affect the development of alloxan diabetes Inhibition of a univalent-cation pump in the B-cell membrane has been suggested to play a role in alloxan diabetes although it need not to be the most basic action of alloxan (*Idahl et al* 1977)

Assuming a direct intracellular action of alloxan it is important to note that certain sulfhydryl reagents specifically inhibit the phosphate carrier of rat liver mitochondria without affecting a number of other mitochondrial functions (*cf Chappell* 1970, *Lehninger* 1975), and that one of the most well-known properties of alloxan is its role as sulfhydryl reagent The B-cell mitochondria like those of liver, are supposed to possess a sulfhydryl dependent phosphate transport system which is inhibited by alloxan (*Lorentzon & Boquist* 1979) This inhibition may result in a decreased mitochondrial content of Pi and an increased concentration of Pi in the cytosol which in turn may be consistent with the increased concentration of Pi in whole islets (*Boquist* 1978 e), and with the altered pattern of pyroantimonate precipitation in the B-cells of alloxan treated mice (*Boquist* 1977a) The alterations in the localization and concentration of Pi may be associated with similar alterations of Ca^{2+}

Since starvation is well known to increase the B cell sensitivity to alloxan, it should be noted that starvation has been reported to alter the activation site for the »phosphate flush« in rat islets (*Asplund & Freinkel* 1978) although this is not directly applicable to the supposed mitochondrial transport mechanism for phosphate

Mitochondrial lesion, and alterations in the localization and concentration of Pi may affect the release of insulin Pi plays a so far, not fully clarified role in insulin secretion Islet stimulation reduces the islet pool of phosphate, the total islet content of phosphate declines as much as 40–50% following glucose stimulation (*Bukowiecki et al* 1979), and Pi may inhibit glucose induced insulin release *in vitro* (*Andersson* 1978) Thus high

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Alloxan inhibits the oxidative phosphorylation of glucose in various tissues (*Bhattacharya* 1955) and abolishes the respiratory stimulation with glucose and decreases the oxidation of glucose in isolated mouse islets (*Gunnarsson & Hellerstrom* 1973) The notion that alloxan inhibits the NAD linked steps of the tricarboxylic acid cycle (*Younathan* 1962) is of interest since a deficient oxidation of oxaloacetate and citrate but an unaffected oxidation of succinate has been reported in isolated mouse islets exposed to alloxan (*Gunnarsson & Hellerstrom* 1973) and since phosphate transport is an obligatory requirement for the net transport of malate α-oxoglurate and citrate (*cf Barritt et al* 1978) It should also be noted that a protection against alloxan toxicity has been found by NAD *in vitro* (*Younathan* 1962) and by nicotinamide *in vivo* (*Lazarow et al* 1950)

Against this background the supposed block by alloxan of a transport system for phosphate in the B-cell mitochondria is believed primarily to give rise to an inhibition of the NAD linked oxidations and oxidative phosphorylation and to an increased cytosolic concentration of Pi which secondarily causes swelling and further functional impairment and finally disintegration of the mitochondria ntact mitochondrial function is necessary for the nergy production and survival of all cells and ence the mitochondrial lesion may be the cause of 3-cell necrosis in alloxan treated animals (*Boquist* 1977 a *Lorentzon & Boquist* 1979)

Because of the finding of mitochondrial lesions in alloxan treated mice the effects on the endocrine pancreas of *p* hydroxymercuribenzoate (PMB) were investigated (*Boquist* 1978 c and 1979 a) since this compound is a relatively specific sulfhydryl reagent with an established inhibitory effect on mitochondrial function *in vitro* suggested to be due to interaction with mitochondrial permeation of phosphate (*Fonyo & Bessmann* 1968) PMB admini ... induced transient hyperglycemia

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Hydrogen Ion Concentration

In contrast to the increased concentration of Pi recorded in whole islets of alloxan treated mice (*Boquist* 1978 e) a decrease was observed in islet pH (*Boquist* 1978 a) Since the islets of the mice used mainly are composed of B-cells data for whole islets can be regarded as representative for these cells Although these data give no information about the localization of ions inside the B-cells they are probably most representative for the cytosol As may be the case in serum there may be an inverse relationship between Pi and pH in the B cells and as in lower organisms (*Harold* 1972) mitochondrial uptake of Pi may result in alkalinization of the exterior *milieu* whereas efflux of Pi may give rise to alkalinization of the mitochondrial interior Therefore the Pi pH hypothesis includes a supposition of an inverse relationship between Pi and pH in the B cells although pH alterations are not necessary to explain the development of mitochondrial lesions and necrosis of B-cells

Changes in hydrogen ion concentration obviously affect B-cell function *in vitro* The net release of H^+ is markedly increased by glucose (*Malaisse et al* 1979) and modifications in extracellular pH may interfere with emiocytosis (*Rebolledo et al* 1978) Reduced pH has been reported to inhibit glucose induced insulin release (*Lernmark* 1971) whereas a rise in pH enhances insulin secretion possibly through a dissolution of secretory granules (*Hellman* 1975) Isolated secretory granules exhibit an optimal stability at pH 6 0 (*Coore et al* 1969)

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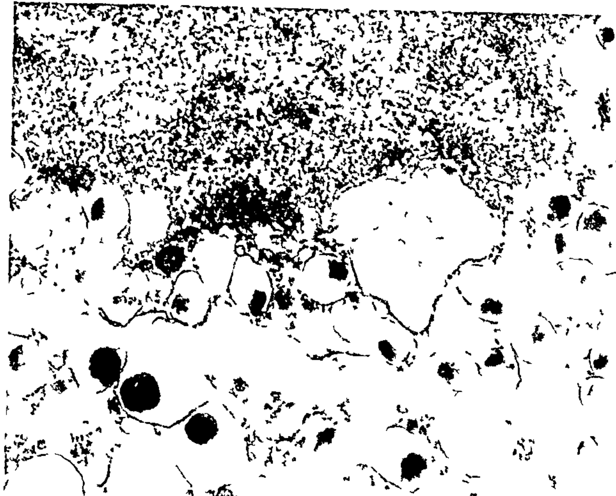


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cytosolic concentration of Pi in the B-cells may contribute to inhibited glucose induced insulin secretion in alloxan treated mice

In vitro studies of mitochondria from some kinds of cells have demonstrated swelling and loss of function in the presence of Pi Thus exposure of rat liver mitochondria to Pi has been reported to be the mildest treatment which causes marked structural changes and inactivates the oxidative and phosphorylative systems (*Hunter & Ford* 1955) The NAD dependent oxidations of the tricarboxylic acid cycle are both inhibited by this kind of treatment (*Hunter & Ford* 1955) and in rat kidney homogenates by exposure to alloxan (*Younathan* 1962) Inhibition of oxidative phosphorylation by some organic mercurial compounds is due to inhibition of the phosphate entry into the mitochondria (*cf Fonyo* 1968) which may regulate oxidative phosphorylation (*Barritt et al* 1978)

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... *in vitro* suggested to be due to interaction with mitochondrial permeation of phosphate (*Fonyo & Bessmann* 1968) PMB administration to fed mice induced transient hyperglycemia and structural B-cell changes similar to those found early efter alloxan treatment (*Boquist* 1978 c and 1979 a) and inhibited insulin release *in vivo* (*Boquist* 1979 d) and *in vitro* (our unpublished observation) However, PMB injection caused no B cell necrosis or any permanent diabetes and was without effect on the blood glucose concentration and B-cell morphology in starved mice Thus although the effects on the endocrine pancreas of alloxan and PMB are not identical the similarities in these effects are sufficiently great to support indirectly the contribution that the PMB-data make to the Pi pH hypothesis

Hydrogen Ion Concentration

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glucose tolerance (*Boquist* 1979 b) This emphasizes that pH alterations mat play a prominent role in B-cell function

It is also of interest that glycolysis in various tissues is influenced by the hydrogen ion concentration Glycolysis in erythrocytes, which may be alloxan sensitive since hemolysis occurs in alloxan diabetes, is inhibited both by high concentrations of Pi (*cf Schrier* 1970) and by acidification (*Tulin et al* 1947), whereas low concentration of Pi and alkalinization have an opposite effect

Against this background it seems that an incrased pH in the B-cell cytosol may facilitate, or be necessary, for insulin release, at least for secretion of insulin stored in granules If so, the inhibited insulin secretion after exposure to alloxan may at least in part be due to decreased pH Moreover, reduced pH is believed to potentiate the action of alloxan in the B-cells, since it is known that alloxan *in vitro* is highly instable in neutral and alkaline solutions, whereas acidity increases its stability, and since *in vivo* studies have disclosed that the alloxan effect is potentiated in rats by acidosis (*Klebanoff & Greenbaum* 1954), but antagonized in mice by metabolic alkalosis (*Boquist* 1979 c) Acidity may even be a prerequisite for alloxan toxicity in the B-cells

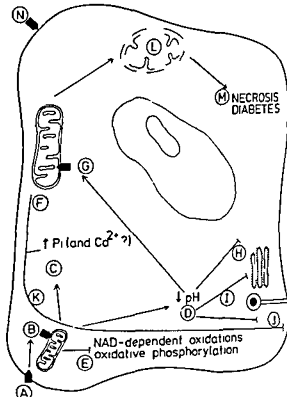


Fig 4 Schematic representation of the Pi pH hypothesis for alloxan diabetes Alloxan (■▶) passes the B-cell membrane through a channel (A) which probably is associated with transport of glucose and possibly also with that of sodium and phosphate and inhibits a mitochondrial sulfhydryl dependent transport system for Pi (B) This results in an increased concentration of Pi [illegible] pH (D) in the cytosolic Pi concentration causes a swelling of the mitochondria (F) The lowering of the pH increases the stability of alloxan (G) and possibly causes a decreased glycolysis (H) and an inhibition of insulin synthesis (I) and the glucose induced release of insulin (J) The mitochondrial changes and the increased concentration of Pi in the cytosol may also contribute to the inhibition of glucose induced secretion of insulin (K) The swollen mitochondria are subsequently disintegrated (L) the energy production ceases and B-cell necrosis and diabetes develop (M) In addition to the effect on the mitochondria there may be a direct or indirect action of alloxan on the plasma membrane (N) The selectivity of alloxan for the B-cell is believed to be associated with the permeability of this cell for glucose

HYPOTHESIS

Summing up (Fig 4), alloxan is believed to pass the plasma membrane of the B-cells, probably through a glucose transport channel, which may be associated also with transport of sodium and phosphate In addition there is possibly a direct or indirect action on the plasma membrane Inside the B-cell alloxan is believed to inhibit a sulfhydryl-dependent trans port system for phosphate in the (inner membrane of?) mitochondria leading to increased concentra tion of Pi in the cytosol and, since an inverse relationship is supposed to exist between Pi and pH secondarily to a fall in pH in the cytosol Secondarily, the localization and concentration of Ca^{2+} in the B-cell may also be affected The block of mitochondrial phosphate transport also gives rise to inhibition of the NAD-dependent oxidations of the tricarboxylic acid cycle and oxidative phospho rylation The increase in cytosolic concentration of Pi causes mitochondrial swelling, which possibly further impairs mitochondrial function The fall in pH in the cytosol increases the stability of alloxan and therefore potentiates and prolongs the action of alloxan Acidity may even be a prerequisite for alloxan toxicity Moreover, the fall in pH inhibits the synthesis and the glucose induced release of

insulin, and causes possibly decreased glycolysis Mitochondrial lesion, and increased concentration of Pi in the cytosol may contribute to inhibition of glucose induced insulin secretion The swollen mitochondria are subsequently disintegrated, which results in absence of ene--- ---duction necrosis of B-cells, and developmen

In order to test the hypothesis studies have been carried out of factors affecting the B-cell sensitivity to alloxan These studies disclosed an inhibition of the development of alloxan diabetes in starved and/or fed mice pretreated with D glucose D mannose D fructose sodium lactate $NaHCO_3$ D mannoheptulose PMB and acetazolamide and in mice given L-leucine before or within 3 min after alloxan injection whereas no effect on the B-cell sensitivity to alloxan was observed after administration of D galactose L arginine NaH_2PO_4 or Ca^{2+} (*Boquist* 1977 a 1978 a b c d e 1979 b c d and e) A potentiation of alloxan toxicity on the other hand was induced by starvation or pretreatment with 1 25 dihydroxycholecalciferol or parathormone (*Boquist et al* 1977)

A rise in islet pH was observed after administration of those substances which are antagonistic to alloxan whereas a fall in islet pH was found after starvation and as described above after alloxan administration (*Boquist* 1978 a) The islet concentration of Pi on the other hand was assayed after exposure to D glucose L-leucine and as described above after administration of alloxan the Pi concentration in the islets was decreased after D glucose and L leucine but increased after alloxan (*Boquist 1978 e*) Thus it appears that alloxan sensitivity is associated with high Pi and low pH and that alloxan protection is associated with low Pi and high pH These findings support the Pi pH hypotesis

A high pH may act antagonistically by decreasing the stability of alloxan and a low Pi may make the mitochondria more resistant to alloxan Conversely a low pH increases the stability of alloxan and a high Pi increases the size of the mitochondria thereby possibly making these organelles vulnerable Mitochondrial hypertrophy and enhanced succinate dehydrogenase activity have been observed in isolated islets exposed to low glucose (*Boquist* 1977 b) which might be associated with high Pi and low pH

Glucose mannose and fructose are the only sugars which can be glycolyzed by most cells (*Villar Palasi et al* 1957) Therefore the reason why pretreatment with these hexoses protect against alloxan may be that they through glycolysis affect Pi and pH in the B-cells This could explain why these hexoses h [illegible]

Mor[illegible]
efflu[illegible]
rat [illegible]
(*Fre[illegible] et al* 1976)

Also L-leucine causes a transitory heightened efflux of Pi from isolated rat islets (*Freinkel et al* 1976) which conceivably decreases the B-cell concentration of Pi thereby antagonizing alloxan toxicity according to the Pi pH hypothesis It should be noted that the effect of L-leucine on Pi efflux is not inhibited by D mannoheptulose (*Freinkel et al* 1976) since pretreatment with L-leucine does not affect the hyperglycemic response of fed mice to D mannoheptulose (*Boquist* 1979 e) In contrast to the other compounds tested L leucine protected also when given a few minutes after alloxan possibly indicating another protective mechanism than for the other compounds tested (*Boquist* 1979 b) One may speculate whether L-leucine passes into the B cells and causes Pi efflux also after these cells have been exposed to alloxan whereas *e g* glucose does not

The metabolic alkalosis induced in mice pretreated with sodium lactate or $NaHCO_3$ (*Boquist* 1979 c) might have decreased the stability of alloxan already when it is present in the circulation Another possibility is that the increase in blood pH was associated with a rise in B-cell pH which inhibited alloxan toxicity intracellularly

The selectivity of the B-cells for alloxan toxicity is believed to be due to the fact that these cells are freely permeable to glucose Also the few other kinds of cells which are sensitive to alloxan are freely permeable to glucose *e g* the kidney tubule epithelial cells Still other cells which are not freely permeable to glucose are resistant to alloxan toxicity The free permeability to glucose may directly or indirectly be associated with a free permeability also for alloxan An alternative explanation is that alloxan induces inhibited insulin release, and that in the absence of sufficient amounts of insulin glucose and Pi (which are supposed to enter the cell in association) pass into those cells [illegible]

[illegible] such accumulation is deleterious to the cell according to the Pi pH hypothesis

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THE DIAGNOSIS OF CARCINOMA IN TRANSURETHRAL RESECTATES OF THE PROSTATE

A Study of the Probability of Overlooking Malignant Tissue when only Part of the Material Is Embedded for Histological Examination

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Rismyhr B Eide T J & Stalsberg H The diagnosis of carcinoma in transurethral resectates of the prostate A study of the probability of overlooking malignant tissue when only part of the material is embedded for histological examination Acta path microbiol scand Sect. A *88* 211–215 1980

The purpose of this retrospective study based on the findings in routinely processed material from fifty two consecutive cases of prostatic carcinomas is a mathematical calculation of the probability of including a carcinoma in the histological sections from tissue obtained by transurethral resection of the prostate when different embedding practices are followed The analysis shows that if a material of a composition similar to ours were examined the calculated probability to include at least one prostatic chip with malignant tissue in the histological sections would be about 91% if one paraffin block is made The probability increases to about 96% when two blocks are used and comes close to 99% with four blocks

Key words Prostate carcinoma transurethral resectates histology overlooking

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The amount of tissue obtained by transurethral resection of the prostate varies considerably from case to case It is common practice in most laboratories not to embed more material for microscopic examination than can be placed in a few paraffin blocks of standard size However in a small proportion of cases malignant tissue is present only in very few tissue chips and the diagnosis of carcinoma will be missed if all of these happen to be in the unembedded part of the material

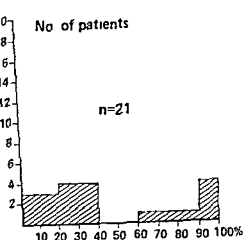
The present study was incited by the report of *Lefer & Rosier* (1976) who found a significant increase in the prevalence of prostatic cancer by embedding about twice as much material for microscopic examination as they did previously

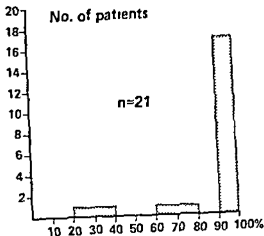
The purpose of this study is a mathematical calculation of the probability of [illegible]ding a carcinoma in the histological sections when different embedding practices are followed The calculations are based on the findings in routinely processed material from 52 consecutive cases of prostatic carcinoma diagnosed histologically in transurethral *resectates*

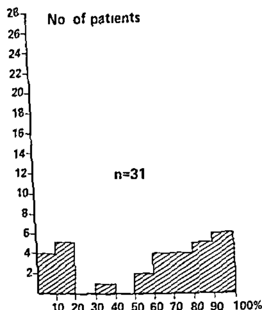
MATERIAL AND METHODS

This retrospective study was performed at the Depart[illegible]

[illegible] The weight of the prostatic material received and the proportion of the material embedded were taken from the reports No fixed embedding rules had been followed during the period but at least 2 or 3







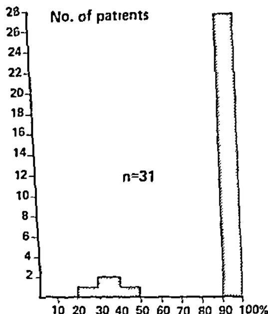


Fig 1 Distribution of patients with clinically benign prostatic disease (upper diagrams) and with clinically malignant prostatic disease (lower diagrams) in 10 per cent intervals according to the proportion of prostatic chips (both left diagrams) and the proportion of paraffin blocks (both right diagrams) containing malignant tissue

malignant diagnosis In the two clinical groups taken together the proportion of malignant to benign chips was less than 1 5 in fifteen cases (29%) less than 1 10 in seven cases (13%) and less that 1 20 in four cases (8%) As seen from the diagrams the distribution of the proportion of malignant chips appears to be biphasic in both clinical groups with one peak frequency at 100% malignant chips and another peak at about 20% malignant chips

Table 2 shows the calculated probability in per cent to include at least one prostatic chip with malignant tissue in the histological sections if a material of a composition similar to ours were examined under different embedding practices It is assumed that the maximum number of blocks is always used except when the total material can be embedded in a smaller number of blocks and that fourteen chips are contained in each block except the last which may have a smaller number If the

plastic casettes (Ames Company No 4187) were filled for embedding when sufficient material was available The tissue was embedded in paraffin and sections were cut at 5 μm and stained with hematoxylin and eosin

One section from each block was used for microscopic examination in the present study Each prostatic chip was marked on the slide with coloured ink for having either malignant or only benign tissue The evaluation was performed by two observers using a discussion microscope The presence of malignancy was based on standard histological and cytological criteria (*Evans* 1978) and each chip was evaluated individually regardless of the presence of malignancy in other chips on the same slide The numbers of chips with and without malignant tissue were counted including only chips with a maximum diameter ≥ 0 5 mm The measurement was performed with an ocular micrometer

The total number of chips present in the material received in the laboratory was estimated from the observed number of chips sectioned and the information on the proportion of the material embedded given in the pathology reports The number of blocks required for embedding of the total material from each patient was calculated from the observed mean number of chips per block in the total material

The probability finding at least one prostatic chip with malignant tissue among a given number of chips (q_n) was calculated by the following formula

$$q_n = 1 - p_1 \; p_2 \qquad p_i \qquad p_n$$

where

n is the number of chips examined and p_1 is the probability that one randomly selected chip does not contain malignant tissue

p_i is the probability that the i th randomly selected chip does not contain malignant tissue when all of the i−1 randomly selected chips also do not contain malignant tissue

p_1 is calculated by

$$p_1 = \frac{B}{T}$$

B is the total number of chips without malignant tissue

T is the total number of chips

p_i is calculated by

$$p_i = \frac{B-(i-1)}{T-(i-1)}$$

RESULTS

The fifty-two patients in whom primary adenocarcinoma of the prostate was microscopically confirmed constituted 19 1% of all patients from whom transurethral resectates of the prostate were received in the department *during the period* under study The clinical diagnosis was benign enlargement of the prostate (CBP) in twenty-one patients and suspected malignant disease of the prostate (CMP) in the remaining thirty-one patients Table I shows the age-distribution of the two categories of patients The mean age of the patients with CBP and CMP was 74 and 73 years respectively

TABLE I *Age-Distribution of Fifty-Two Patients with Prostatic Carcinoma in Transurethral Resectates*

Age-groups	Clinical diagnosis		Total
	Benign	Malignant	
50–59	3	1	4
60–69	3	8	11
70–79	8	13	21
80 +	7	9	16
All ages	21	31	52

The weight of the material received was known in twenty-five cases and ranged from 1 to 30 g with a mean of 11 9 g From the frequently rather rough estimates regarding the proportion of the material that had been embedded for microscopic examination it was estimated that an average of 69% of the material had been embedded in cases of CBP and 81% in cases of CMP The mean number of paraffin-blocks prepared was 3 4 in cases of CBP and 2 6 in cases of CMP The mean number of prostatic chips counted in one section from a block was 14 2 and was similar in both categories of patients

Fig 1 shows the distribution of patients according to the proportion of prostatic chips and the proportion of paraffin blocks that contained malignant tissue The results are given separatly for patients with clinically benign (upper diagrams) and clinically malignant (lower diagrams) prostatic disease

In forty-four of the fifty-two cases, all blocks contained malignant tissue Of the remaining cases malignant tissue was present only in half of the blocks or less in six There was no significant difference between the two clinical groups in this respect

The proportion of individual chips that contained malignant tissue was on the average 42% among patients with a clinically benign diagnosis and 59% among patients with a clinically malignant diagnosis As might have been expected it was relatively more common to find only a small proportion of chips with malignant tissue in cases with a clinically benign diagnosis, but such findings also occurred in a substantial number of cases with clinically

of latent microcarcinomas A follow-up study is planned to answer this question

In any case, one would wish to have a reasonably high probability of diagnosing a carcinoma when this is present in the material received in the laboratory, and we would recommend that at least 4 blocks of tissue are embedded when such amounts of tissue are received Further studies will show whether the proportion of malignant tissue present is of sufficient clinical importance to recommend that it should be stated in the pathology report

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TABLE 2 Calculated Probability in per cent to Include at Least One Prostatic Chip with Malignant Tissue when This Is Present in the Resected Material According to the Maximum Number of Blocks Embedded and According to Clinical Diagnosis

No of blocks	Clinical diagnosis		Total
	Benign	Malignant	
1	91.5	91.3	91.4
2	95.8	95.9	95.9
3	97.2	98.4	97.8
4	98.0	99.5	98.9
5	98.5	99.9	99.3
6	98.9	99.9	99.5
7	99.2	99.9	99.6
8	99.5	99.9	99.7

practice is that only one paraffin block is made the average probability including at least one prostatic chip with malignant tissue would be about 91% regardless of clinical diagnosis The probability increased to about 96% when two blocks are used and comes close to 99% with four blocks

DISCUSSION

Macroscopic assessment of transurethral resected prostatic material for malignancy is time-consuming and unreliable (*Lefer & Rosier* 1976 *Medart* 1977) and in most cases the prostatic chips embedded for microscopic examination are taken by chance When the presence of malignant tissue is limited to a small proportion of the chips received in the laboratory the probability of including a carcinoma is therefore dependent on how much of the resected material that is embedded *Lefer & Rosier* (1976) in two series over two periods found an increase in prevalence of carcinoma in transurethral resected prostatic material from 8% in the first series to 14% in the second series The average number of blocks per patient in the two series was 2.8 and 5.4 respectively and they concluded that the difference in prevalence of carcinoma was due to a more thorough examination of the specimen in the second series Also *Medart* (1977) found a higher frequency of prostatic carcinomas as he changed from the processing of only two or three blocks to the processing of the entire specimen

Most patients in our study did not have malignant tissue in all prostatic chips and 17% of the patients did not have malignant tissue in all blocks If a material of resectates similar to ours had been examined by embedding one block contain fourteen chips from each patient malignant tiss would be expected to be present in the histolog section in 91% of the cases This would increase 96% of the cases if two blocks were made and 99% if four blocks were made There is lit difference in this respect between patients with clinically benign diagnosis and those with clinically malignant diagnosis in our material

The validity of these figures has certain limit tions The number of chips in each block has bee counted from the routine histological section which may not have included chips lying deep in th block On the other hand both ends of curved chi may have been cut and appeared as two separa pieces of tissue on the slide The calculation of probability is based on the cases in which th histological diagnosis of carcinoma was made in th current routine of the department and a few case in which malignant tissue has been present in only few chips may well have been missed This numbe cannot be calculated from the present data and prospective study in which all chips are embedde and sectioned will be needed to give reliable data o this point The presence of such cases however means that our estimates of the average probability of including a carcinoma when present in the received material are too high and that more blocks will be needed to reach a given level of probability Also cutting each block at several levels might be expected to reveal more cases with sparse amounts of malignant tissue and this would further change the probability estimates in the same direction

However the clinical significance of the presence of small amounts of carcinomatous tissue in transurethral resectates is not known Theoretically the presence of a few malignant chips may be due to a clinically important carcinoma that is located in the peripheral part of the prostate and mostly outside the part of the gland removed by operation or it may be due to a latent micro-carcinoma Autopsy studies have shown latent micro-carcinomas of the prostate to be present in about 40% of all men over 60 years of age (*Harbitz & Haugen* 1972) and although most of these were located peripherally some were in a more central position It must be assumed therefore that these clinically insignificant lesions which cannot be distinguished from other prostatic carcinomas by histological criteria appear in a certain number of transurethral resectates that are removed because of symptoms produced by benign prostatic hyperplasia The biphasic distribution of the frequency of malignant chips in resectates in our material is striking and it may be questioned whether the low frequency peak could be largely produced by the incidental inclusion

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INTESTINAL METAPLASIA WITH COLONIC-TYPE SULPHOMUCINS IN THE GASTRIC MUCOSA; ITS ASSOCIATION WITH GASTRIC CARCINOMA

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Sipponen P Seppala K Varis K Hjelt L Ihamaki T Kekki M & Siurala M Intestinal metaplasia with colonic type sulphomucins in the gastric mucosa its association with gastric carcinoma Acta path microbiol scand Sect. A 88 217–224 1980

The occurrence of intestinal metaplasia with colonic type sulphomucins (abbreviated s IM or s positive IM) in the gastric mucosa was studied in 125 patients with gastric carcinoma (GCA) 62 patients with pernicious anemia (PA) 301 and 183 first-degree relatives of GCA and PA patients 406 outpatients and in 358 controls matched from a large population sample The sulphomucins (s mucins) were demonstrated histochemically in endoscopic biopsy specimens by using high iron diamine and Alcian blue (pH 1) methods The prevalence of s IM especially the strongly s positive IM was significantly higher in GCA patients than in other series or controls of the same age group Further s IM was significantly more common in PA patients and its prevalence higher in GCA relatives and outpatients than in other series or controls The occurrence of s IM was also age dependent and dependent on the extent of IM It was suggested that the occurrence of s IM can be used as a sign of lesions that are more closely associated with GCA than IM is in general

Key words Gastric carcinoma intestinal metaplasia premalignant lesions carcinogenesis histochemistry

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Several studies tentatively indicate a causal interrelationship between intestinal metaplasia (IM) and gastric carcinoma (GCA) (2 6 10 13–16 18) The prevalence of IM is higher in GCA than in benign gastroduodenal diseases (3 6 10) and there exist all transitional forms between IM and GCA (16 19–22 24) In addition the cancerous epithelium itself displays morphological and histochemical characteristics of intestinal epithelium (1 4 5 8 12 23 25 28)

Some IMs seem to contain mucins with sulphated residues characteristic of the colonic epithelium while some of the IMs share only the characteristics of the small bowel epithelium (4 8 12 29) According to a few recent studies the »colonic type IM« is more closely related to GCA than IM is in general (6 7 29) These studies however are based upon examination of surgical specimens obtained at operations for GCA and benign gastroduodenal ulcer but no data are available on the occurrence of the »colonic type IM« in less selected and more representative materials and in conditions commonly considered precancerous We have studied in this investigation the occurrence of IM with colon like sulphomucins in antral and body mucosa of the stomach in endoscopic biopsies from patients with GCA or pernicious anemia (PA) from first-degree relatives of GCA or PA patients and from a consecutive series of patients gastroscopized in the out patient department of a hospital A large sample

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of the general adult population of Finland has been used as reference material The study was aimed to clarify whether IM with colon like sulphomucins is significantly associated with GCA and whether its prevalence is higher in precancerous conditions such as pernicious anemia and in first-degree relatives of GCA or PA patients

MATERIALS AND METHODS

Materials

The following materials were studied

– *Series I* consists of 125 consecutive gastric carcinoma patients diagnosed at the gastroenterological units of Jorvi and Meilahti hospitals

– *Series II* consists of 62 consecutive pernicious anemia patients treated at the Outpatient Department of Meilahti hospital

– *Series III* consists of 301 first-degree relatives of 73 consecutive patients with gastric carcinoma diagnosed at the Gastroenterological Unit of Meilahti Hospital

– *Series IV* consists of 183 first degree relatives of 68 consecutive pernicious anemia patients

– *Series V* consists of 406 consecutive patients without gastric carcinoma studied by direct vision gastric biopsy at Jorvi hospital The series includes 17 patients with active gastric ulcer and 19 patients with active duodenal ulcer and approximately 200 patients with other circumscribed endoscopic lesions such as polyps erosions atrophy etc

– *Control series* consists of 358 first degree relatives of subjects computer matched from general population according to age and sex for 73 carcinoma patients of series III

The mean age and sex distribution of the series is given in Table 1 More data on the series and controls are given elsewhere (9 30)

Biopsy Procedure

Using the direct vision gastroscopic biopsy method 2–4 specimens were obtained from the antrum mucosa and 6–10 from the body mucosa in series II–IV and in controls In series I and V approximately 2 specimens were obtained from both antrum and body by employing a similar method In series I ? additional specimens were obtained from the cancerous area

Histochemical Methods and Interpretation of Staining Results

Biopsy specimens were fixed overnight in neutral buffered 10 per cent formalin and were embedded in paraffin All tissue sections were stained by the haematoxylin-eosin and Alcian blue (pH 2 5)-PAS methods The occurrence of sulphomucins in IM cases [illegible] at pH 1 (AB1) (17) [illegible] ods The results of [illegible] rarily as + + + [illegible] action which was [illegible] easily distinguished from the background were graded + and those with an intense staining reaction corresponding the staining intensity of normal colonic mucosubstances were graded + + Examples for the grading are presented in Figs 1 and 2 The patients and subjects were grouped according to the highest grade in any of the specimens studied

When the sections were cut for the histochemical studies it sometimes appeared however that the metaplastic foci were not present in the new sections and accordingly their identification was therefore not possible These cases were excluded from the IM group

The extent of IM in tissue sections was graded arbitrarily as + + + or + + + The cases with a single metaplastic gland were graded + and those with extensive IM corresponding the extent in total atrophy were graded + + +

Statistics

Statistical significances were calculated by using Student s t or χ^2 tests

RESULTS

Examples of foci with intestinal metaplasia (IM) with or without sulphomucins (abbreviated s IM – s IM + or s IM + + or s negative IM or s positive IM respectively) are presented in Figs 1 and 2 Sulphomucins were seen in goblet cells and occasionally in the brush border of the metaplastic glands (Fig 1) The staining intensity of the goblet cells showed a large variability even within the same gland This variability was most remarkable in cases with an intense staining reaction (s IM + +) in which even non goblet cells were occasionally stained (Fig 2)

The total prevalences of IM in series and controls are presented in Table 1 The total prevalence of IM is higher in patients with gastric carcinoma (GCA) and pernicious anemia (PA) than in other groups It also appears however that the mean ages in series III V and in controls are similar but differ from those in series I and II This necessitates a comparison of series I and II with an age and sex matched sample of the controls These calculations revealed the total prevalence of IM was significantly higher ($p < 0.01$) in GCA and PA patients than in their matched controls In addition the corresponding prevalences in GCA relatives and in outpatients were also significantly ($p < 0.05$ and 0 01 respectively) higher than those in controls

Table 2 shows the occurrence of IM with sulphomucins (s IM) in series and controls The prevalence of s positive IM is significantly ($p < 0.01$) higher in GCA patients than in matched controls Also the prevalence of s positive PA patients is significantly ($p < 0.01$) higher than that in matched controls However no such significant

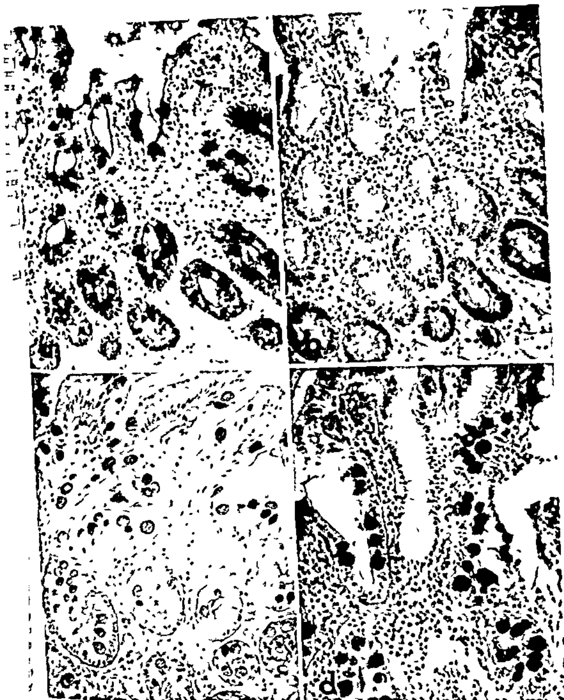


Fig 1 Examples of intestinal metaplasia with and without sulphated colonic type mucosubstances A IM foci stained with Alcian blue (pH 2 5)–PAS B same area as in Fig A stained with high iron diamine (HID) Note the absence of stainable mucosubstances (s IM –) C IM foci stained with HID Note the staining of goblet cells and a slight staining in the brush border (s IM +) D IM foci with intense staining of mucosubstances with HID (s IM + +) × 300



Fig. 2 Intense staining of mucosubstances with HID (s IM + +) A dysplastic glands stained with Alcian blue (pH 2.5)–PAS B same area stained with HID Note the intense staining of mucosubstances also in non goblet epithe cells × 300

TABLE 1 *Mean Age Male Female Ratio and Prevalence of Intestinal Metaplasia (IM) in the Patient Series St*

Series	Total Number of subjects	Mean Age yrs ± S D	Male Female Ratio	Subjects with IM	
				Number	Per cent
(I) GCA Patients	125	65 ± 12	1.8	61	49
(II) PA Patients	62	65 ± 14	0.4	41	66
(III) GCA Relatives	301	47 ± 13	0.9	60	20
(IV) PA Relatives	183	47 ± 16	0.8	24	13
(V) Outpatients	406	52 ± 15	0.9	141	34
Controls	358	46 ± 14	0.9	50	14

TABLE 2 *Prevalence of Intestinal Metaplasia with Sulphated Colon Type Mucosubstances (s IM) in the Patient Series*

Series	Total Number of Subjects	Subjects with s IM					
		s IM + No	%	s IM + + No	%	s IM Total No	%
(I) GCA Patients	125	24	19	19	15	43	34
(II) PA Patients	62	10	16	1	2	11	18
(III) GCA Relatives	301	25	8	5	2	30	10
(IV) PA Relatives	183	6	3	0	–	6	3
(V) Outpatients	406	36	9	4	1	40	10
Controls	358	19	5	3	1	22	6

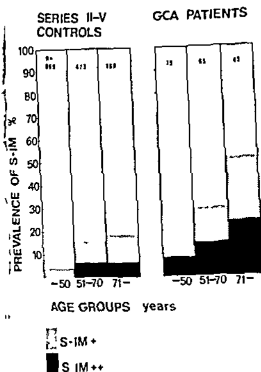


Fig 3 Prevalence of intestinal metaplasia with sulphated colon like mucosubstances (s IM) in patient series in relation to age Controls and patient series without malignancy are treated as one group

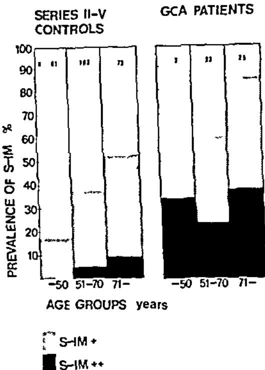


Fig 4 Prevalence of intestinal metaplasia with colon like sulphated mucosubstances (s IM) in patients with intestinal metaplasia Controls and patient series without malignancy are treated as one group

differences are seen between PA relatives and controls On the other hand the prevalence of s IM is higher in GCA relatives and in outpatients than in controls although this difference is not statistically significant ($0.1 > p > 0.05$)

The age relation of s IM is shown in Figs 3 and 4 Because of the small number of s positive cases all series without carcinoma are treated as one group and are compared with the carcinoma series It appears that in both groups the prevalence of s positive IM increases with age The data further reveals that the proportion of s positive IM of all metaplasias increases also with age (Fig 4) However in all age groups the prevalence and proportion of s positive IM was higher in GCA patients than in the non-cancer group

In agreement with the above results the mean age in subjects without carcinoma tends to be higher in

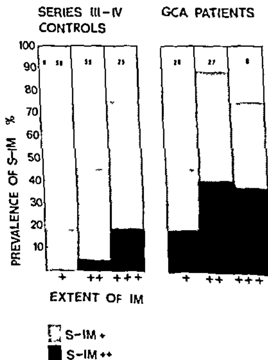


Fig 5 Prevalence of intestinal metaplasia with colon like sulphated mucosubstances (s IM) in relation to extent of IM The comparison is made between series in which the number of biopsy specimens is the same (controls and series III–IV are compared with carcinoma patients)

relation to the occurrence of s-positive IM If series II–V and controls are treated as one group, the *mean age of strongly s positive subjects* (s-IM + +) is significantly higher ($p < 0.01$) than those in *slightly s-positive IM* (s-IM +) and s-negative IM (s-IM −) cases (mean ages 68 ± 5, 62 ± 14 and 59 ± 13 years, respectively)

The relationship of s-positive IM with the extent of IM is presented in Fig 5 The prevalence of s-positive IM tends to increase with increasing extent of IM in subjects without carcinoma In carcinoma patients, however, this relationship is less pronounced, and carcinoma cases were seen in which even some single metaplastic glands could show an intense staining reaction for sulphomucins

DISCUSSION

Intestinal metaplasia (IM) is a common finding in biopsy specimens from the stomach We have found IM in about 20 per cent of subjects matched from a population sample representative of the general adult population of Finland (26) Although IM has been suggested to be a premalignant condition, its common occurrence inhibits its use as a marker of threatening malignancy The heterogeneity of IM has been known for a long time (1, 12, 23, 35) Some reports have been published in recent years which suggest that IM with colon-like characteristics is more closely associated with gastric carcinoma than IM without these characteristics (6 7 29) In this study, we have used histochemical mucin stains in order to differentiate metaplasia into those containing colon like sulphated mucosubstances (here abbreviated s positive IM or s-IM) and those which do not have these characteristics (abbreviated s negative IM or s IM −) We studied the association of s positive IM with gastric carcinoma and tried to clarify the relationship of s-positive IM to factors that could indicate its premalignant nature

We were able to demonstrate transitional forms between s-negative IM and strongly s-positive IM (s-IM + +) In addition the present data showed that s positive IM is clearly age dependent and is related to the extent of IM In agreement with these results it was found that the mean age of subjects with strongly s positive IM was significantly higher than that of s-negative subjects These results seem to indicate that with respect to s-negative and s-positive IM we are dealing with different stages of the same process rather than with two qualitatively different phenomena Whether the end stage of this process is gastric carcinoma cannot, however be proven by the present study

In gastric carcinoma s positive IM (especially s-IM + + cases) was found significantly more often than in any other series studied also when comparison was made with age- and sex matched controls The results are well in line with those obtained by other investigators (6, 7, 29) and suggest some relationship between s positive IM and gastric carcinoma The results are also in line with the initial observation of *Järvi and Laurén* (10) that a major part of gastric carcinomas show morphogenesis through s c intestinalization It is therefore, understandable that colon like characteristics are also seen in benign lesions that possibly are precancerous, such as IM

There appeared also some association between s positive IM and overt pernicious anemia (PA) or first-degree relatives of gastric carcinoma (GCA) which are often considered to be premalignant states (2, 9, 26, 30) The prevalence of s positive IM was significantly higher in patients with PA and higher in GCA relatives than in controls age and sex matched from a population sample S IM could therefore, be a factor which links PA and GCA relatives with GCA On the other hand there are many morphological and functional similarities between these states other than s-IM which might also explain the higher risk of patients to develop GCA (26, 30) It should be also noted that we could not find differences in the occurrence of s IM between controls and PA relatives, who also have been considered gastric carcinoma risk subjects (26 30) However according to *Lehtola* (11) the risk of gastric carcinoma among first degree relatives of GCA patients is only less than twofold higher than in the population at large Accordingly, even if we assume that s-IM is responsible for the increased risk among GCA relatives the expected difference between GCA relatives and controls cannot be very great Indeed there was a difference in this respect the prevalence of s-IM in GCA relatives was nearly twofold that in the controls although this difference was not statistically significant

In spite of the association of s-IM with GCA and some precancerous states the present investigation can give only tentative hints as to relationship between s-IM and GCA The results do not exclude either the possibility that GCA is a primary lesion in relation to s-IM or the possibility of mere coincidence It is hardly possible to solve this problem by a simple cross sectional study A adequate long term follow-up is necessary, and such a study has already been started by our team

The limitations of the present study lie also in the methodology employed Although in most series 1(or more biopsies were obtained from each subject the specimens still represent only a small fraction o

in the whole gastric mucosa This means that IM and IM could have been overlooked in some cases In addition when new sections were cut the IM originally present could no longer be found in some specimens It is therefore probable that the presented data on the prevalence of IM and s IM do not express the actual values but are considerably

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ALPHA-1-ANTITRYPSIN GLOBULES IN LIVER BIOPSIES

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Clausen Per Prætorius Alpha 1 antitrypsin globules in liver biopsies Acta path microbiol scand Sect A *88* 225 230 1980

In order to examine the frequency of alpha 1 antitrypsin (AAT) deficiency of phenotype Pi Z in a consecutive liver biopsy material PAS/diastase resistent globules with positive immunohistochemical reaction for AAT (AAT globules) were used as a marker of the Pi Z gene 34 (4%) of 850 liver biopsies contained AAT globules More than half of the biopsies with globules had chief histological diagnoses within the groups fibrosis suspicion of cirrhosis and cirrhosis Micronodular cirrhosis was significantly more frequent in biopsies with AAT globules The results support the assumption that AAT deficiency of phenotype Pi Z as well in homozygous as heterozygous form is associated with development of liver cirrhosis

Key words Alpha 1 antitrypsin deficiency alpha 1 antitrypsin globules cirrhosis liver biopsies

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The association between liver disease and α_1 antitrypsin (AAT) deficiency of phenotype ZZ was first described by *Sharp et al* in 1969 (21) An increased frequency of juvenile cirrhosis was found among children with phenotype ZZ and further more the presence of intracytoplasmic diastase resistent PAS positive globules (PAS/DIA globules) was described in liver tissue from these patients The PAS/DIA globules have subsequently by immunohistochemical and biochemical methods been shown to contain AAT (12 19) The globules always seem to be present in patients with phenotype ZZ but occur in varying amounts and are sometimes lacking in biopsies from patients with the he[illegible]

[illegible] it is still uncertain to what extent the phenotype MZ predisposes to development of liver disease (2 11 15)

A few papers based on consecutive autopsy materials have used the occurrence of PAS/DIA globules with positive immunohistochemical reactions for AAT (AAT globules) as a morphological marker for the Pi Z gene (2 10 16 18) The occurrence of AAT globules in a consecutive liver biopsy material has however not previously been described

The aims of the present study have been to examine the frequency of AAT globules in a consecutive liver biopsy material and to compare morphological changes in biopsies with and without globules

MATERIALS AND METHODS

The investigation is based on 919 consecutive liver biopsies received in the period 01 01 –30 11 1977 at the Department of Pathology Hvidovre Hospital in Copenhagen 55 rebiopsies and 14 unsuitable biopsies were excluded from the material The material described below thus consists of 850 biopsies from 850 patients

The biopsies were fixed in 10% buffered formalin for 1–4 hours and following routine processing at room temperature embedded in Paraplast® at 59 °C 5 μm serial sections were cut and for the morphological evaluation stained by the following methods hematoxylin-eosin, van Gieson, Masson trichrome, reticulin staining a m Gordon and Sweets, staining for iron pigment a m Perls, PAS staining with and without pretreatment with diastase and orcein supplemented by immunoperoxidase staining for HB_sAg (6)

Furthermore, the biopsies were stained by immunohistochemical technique in order to demonstrate AAT in the tissue Indirect peroxidase labelled antibody technique described elsewhere was used (5)

The following antisera were used 1) Rabbit antihuman AAT (DAKO, Denmark, code no 10 012, lot 017 (titer SRI 0 7 g/l, immunoglobulin concentration 11 g/l)) used in dilution 1 40 2) Peroxidase labelled swine-antirabbit IgG (DAKO, code no P 2190, lot 057, (immunoglobulin concentration 1 3 g/l molar peroxidase/antibody ratio 0 8)) used in dilution 1 20

For control stainings anti-AAT antiserum absorbed with AAT was used in dilution 1 40 Furthermore, the immunoglobulin fraction of serum from non immunized rabbits (DAKO code no X 903, immunoglobulin concentration 20 g/l) was used in dilution 1 80 In addition the primary antiserum was replaced by phosphate buffered saline

All biopsies in which PAS/DIA globules of at least 1 μm could be demonstrated in the cytoplasm of hepatocytes and where in the same cells positive immunoperoxidase staining for AAT could be demonstrated corresponding to the globular rim as well as in granular form were recorded as biopsies with AAT globules

The size of globules in the individual biopsy was estimated and according to the maximum size of the globules in the biopsies the positive biopsies were classified in two groups One with globules up to 3 μm and one with globules larger than 3 μm

Phenotyping of the serum from the patients was not available during the period of investigation

For the statistical evaluation the chi^2 test was used The limit for type I error was set at 0 05

RESULTS

Of the 850 patients 486 were male and 364 female In 34 of the 850 biopsies or 4% AAT globules were found The distribution of sex and age of patients with and without AAT globules is shown in Table 1 No significant difference was observed in the distribution neither of sex nor age in the two groups ($p > 0\ 1$)

The chief histological diagnoses of biopsies with and without globules are shown in Table 2

76 biopsies were morphologically normal In 5 of these or 6 6% AAT globules were present The frequency of biopsies with normal morphology showed no significant difference in the groups of biopsies with and without globules ($0\ 8 > p > 0\ 7$).

Neither did the frequency of biopsies with non specific changes and granulomas in the two groups show any difference ($0\ 7 > p > 0\ 5$), while a significantly increased frequency of acute viral hepatitis was found in biopsies without globules ($0\ 05 > p > 0\ 02$)

More than half of the biopsies with AAT globules had morphological changes according to the diagnosis fibrosis, suspicion of cirrhosis and cirrhosis hepatis Comparing the groups of biopsies with and without globules no significant difference was found in the frequency of fibrosis and morphological changes suspicious of cirrhosis

In contrast significantly more biopsies with cirrhosis were found in biopsies with than without globules ($0\ 02 > p > 0\ 01$) 8% of all biopsies with cirrhosis contained AAT globules The increased frequency of cirrhosis in biopsies with globules was due to a highly significant increase in micronodular cirrhosis ($0\ 01 > p > 0\ 001$), as no difference in frequency was found as regards macronodular cirrhosis ($0\ 99 > p > 0\ 98$)

In order to investigate to what extent supplementary aetiological factors could be demonstrated with

TABLE 1 *Distribution of Sex and Age in Patients with and without AAT Globules*

Age (years)	15–20	21–30	31–40	41–50	51–60	61–70	71–80	≥ 81	Total
Sex	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	
No of patients without AAT-globules	14 16	82 43	78 32	69 50	100 69	84 64	30 63	7 15	816
No of patients with AAT-globules	1 0	1 2	2 1	4 1	7 0	5 7	2 1	0 0	34

TABLE 2 *Chief Histological Diagnosis of Biopsies with and without AAT Globules*

Chief histological diagnosis	Biopsies without AAT globules		Biopsies with AAT globules	
Liver without pathological changes	71	(8 7%)	5	(14 7%)
Liver with non specific changes	101	(12 3%)	3	(8 8%)
Liver with granuloma	6	(0 7%)	0	
Acute viral hepatitis	120	(14 7%)	0	
Chronic aggressive hepatitis	5	(0 6%)	0	
Chronic persistent hepatitis	17	(2 1%)	0	
Hepatitidis sequelae	7	(0 9%)	0	
Primary biliary cirrhosis	3	(0 4%)	0	
Alcoholic hepatitis	6	(0 7%)	0	
Cholestatic hepatitis (incl drug lesion)	19	(2 3%)	0	
Liver with fibrosis	59	(7 2%)	4	(11 8%)
Obs cirrhosis	38	(4 7%)	3	(8 8%)
Cirrhosis (micronodular)	83	(10 2%)	10	(29 4%)
Cirrhosis (macronodular)	37	(4 5%)	1	(2 9%)
Fatty liver	122	(15 0%)	3	(8 8%)
Large duct obstruction	55	(6 7%)	4	(11 8%)
Malignant tumour primary	7	(0 9%)	1	(2 9%)
Malignant tumour secondary	37	(4 5%)	0	
Haemosiderosis	9	(1 1%)	0	
Amyloidosis Dubin Johnson	3	(0 4%)	0	
Sinusoidal dilatation	11	(1 3%)	0	
Total	816	(100%)	34	(100%)

different frequency in biopsies with cirrhosis with and without globules a semiquantitative morphological evaluation has been performed as regards the presence of the following morphological features Fat iron pigment bile pigment alcoholic hepatitis parenchymal activity centrilobular fibrosis bile duct proliferation chronic aggressive hepatitis and Hb_sAg in liver tissue

The most essential results of this investigation are shown in Table 3 None of the mentioned featured showed any difference in frequency in biopsies with and without globules ($p > 0\ 05$)

The relation between the maximum size of the globules and the chief histological diagnosis is shown in Table 4 Biopsies containing globules larger than 3 μm were mainly found in the diagnostic groups Fibrosis suspicion of cirrhosis cirrhosis and hepatocellular carcinoma

TABLE 3 *Morphological Features in Biopsies with Cirrhosis with and without AAT Globules*

	Fat		Iron		Parenchymal activity		Alcoholic hepatitis		Hb_s-Ag in liver tissue		Chronic aggressive hepatitis	
	0	+	0	+	0	+	0	+	0	+	0	+
No of biopsies with AAT globules	11	0	11	0	7	4	8	3	11	0	10	1
No of biopsies without AAT globules	94	26	113	7	98	22	101	19	117	3	119	1

TABLE 4 *Relation between Chief Histological Diagnosis and Maximum Size of AAT-Globules*

Chief histological diagnosis	No of biopsies with AAT-globules	
	≤ 3 μm	> 3 μm
Liver without pathological changes	4	1
Liver with non-specific changes	3	0
Liver with fibrosis	2	2
Suspicion of cirrhosis	2	1
Cirrhosis (micronodular)	6	4
Cirrhosis (macronodular)	0	1
Fatty liver	2	1
Large duct obstruction	4	0
Hepatocellular carcinoma	0	1

DISCUSSION

Since the first description of presence of AAT globules in liver tissue from patients with phenotype ZZ several papers have described the presence of globules in liver tissue from patients carrying the Pi-Z gene as well in homozygous as heterozygous form (review see 20)

The need of supplementary immunohistochemical staining for AAT in the characterization of PAS/DIA globules is emphasized by the fact that PAS/DIA globules without immunohistochemical reaction for AAT have been demonstrated in patients with normal phenotype (MM) (23) and are not rarely found in autopsy materials (17, 18)

The specificity of AAT globules as a morphological marker of the Pi-Z allel has been questioned in a few recent papers (4 13) describing the presence of AAT globules in liver tissue from patients carrying the allels Pi-M and Pi-S A more detailed investigation correlating the serum phenotypes with the histochemical and immunohistochemical staining patterns is thus needed in order to state the specificity of AAT globules as a marker of Pi-Z gene The above mentioned cases are however few in comparison to the great number of cases describing AAT globules in Pi Z patients For all practical purposes the presence of AAT globules thus seems to be rather specific as a morphological expression of the presence of the Pi-Z gene The size of the globules has previously only been specified to a limited extent In one paper only globules up to at least 3 μm were recorded (2) A previous investigation (14) has, however, shown that at least 1 μm globules were compatible with the presence of Pi-Z gene, for which reason 1 μm was chosen as the lower limit in the present investigation

The frequency of AAT-globules in liver tissue has until now only been investigated to a limited extent in unselected materials and only in autopsy material *Eriksson et al* (10) examining 700 consecutive autopsies found AAT globlues in 3 7% of the patients *Reintoft* (16) and *Blenkinsopp & Haffenden* (2) demonstrated AAT globules in respectively 6 3% and 3 6% of the autopsies These frequencies are in accordance with the distribution of phenotypes in populations in Scandinavia (8) and England (7) where the frequency of the phenotype MZ is 3–4% and ZZ less than 1 0/00 The frequency of AAT globules in liver biopsies does not seem to have been systematically investigated previously *Eriksson et al* (10) mentioned that the frequency in an unpublished investigation was found to be 1 %, while *Blenkinsopp & Haffenden* (2) stated that the frequency in biopsies was lower than found in autopsy material without indicating any figures

I have in the present investigation found a frequency of AAT globules in liver biopsies of 4% This frequency is thus in accordance with the above mentioned figures in autopsy material but larger than recorded previously in biopsy materials The difference is probably to a large extent due to the fact that globules in biopsies often are smaller and more sparse for which reason they often are overlooked unless supplementary immunohistochemical staining for AAT is used

While rather widespread agreement exists concerning the correlation of phenotype ZZ to liver cirrhosis and probably hepatocellular carcinoma it is still not clarified whether phenotype MZ is connected to the development of liver disease to a greater extent than other phenotypes *Morin et al* (15) using phenotyping of 132 patients with alcoholic cirrhosis and 37 patients with cryptogenic cirrhosis did not find an increased frequency of phenotype MZ in comparison to a control group of healthy blood-donors *Fisher et al* (11) measuring the AAT serum concentration and using selected phenotyping of patients with decreased serum concentration could not demonstrate an increased frequency of Pi Z heterozygous with cirrhosis in comparison to the controls

In contrast *Eriksson et al* (10) with significantly increased frequency found cirrhosis and liver fibrosis in patients with AAT globules *Blenkinsopp & Haffenden* (2) found in 64 autopsies with cirrhosis 10 patients with AAT globules while only 4 of 110 control patients had globules Seven patients with cirrhosis and globules had macronodular cirrhosis

In accordance with the two last mentioned investigations I have in the present investigation

found a significantly increased frequency of cirrhosis in patients with AAT globules in the biopsies In contrast to previous descriptions (2 23) I have found a predominance of micronodular cirrhosis (all nodules smaller than 1 mm) in cirrhosis with AAT globules Macronodular cirrhosis was not found more frequently in biopsies with than without globules

The discrepancies between the above mentioned results are probably to some extent due to differences in the methods used to examine the frequency of Pi Z gene In one paper (11) the serum concentration of AAT was used as a primary screening method As the serum concentration of AAT is normal in many heterozygous (MZ) patients (22) the stated frequency of heterozygous was

AAT globules cannot be interpretated until the specificity of AAT globules as a marker of the Pi Z gene has been investigated

Previous papers (2 3 23) have investigated to what extent other aetiological factors could be demonstrated to be of importance for the development of liver disease in patients with AAT deficiency *Triger et al* (23) did not find any morphological indications of an increase in alcoholic hepatitis or hepatitis B infection *Blenkinsopp & Haffenden* (2 3) demonstrated Hb_sAg in 4 out of 15 cases of cirrhosis with globules In the present investigation no increase in alcoholic hepatitis or Hb_sAg was found when comparing biopsies with cirrhosis with and without globules

The higher frequency of acute viral hepatitis in patients without globules could be due to a greater resistance of Pi Z patients to viral hepatitis It is however more likely that liver cells with globules are more sensitive to viral induced tissue damage In this way globules could be more difficult to demonstrate

It is noteworthy that the major part of biopsies without primary parenchymal changes contained globules smaller than 3 µm while globules larger than 3 µm mainly were seen in biopsies with fibrosis and cirrhosis

The results of the present investigation lend support to the previously mentioned assumption that both homozygous and heterozygous Pi Z patients are predisposed to the development of liver cirrhosis Further studies are however needed in order to determine the specificity of AAT globules as a morphological marker of AAT deficiency

I wish to thank *Annelise Petersen* and *Niels Johansen* for technical assistance

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CYTOPLASMIC EFFECTS OF X-IRRADIATION ON CULTURED CELLS IN A NON-DIVIDING STAGE

4 *Studies of Sparsely Grown Serum-Deprived Cells*

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Hamberg H Brunk U T Ericsson J L E & Jung B Cytoplasmic effects of X irradiation on cultured cells in a non-dividing stage 4 Studies of sparsely grown serum-deprived cells Acta path microbiol scand Sect A *88* 231–239 1980

Cultured human glial cells were blocked in interphase (G_1) by 24 h of serum starvation and subsequently subjected to 200 Gy 8 MV X radiation Immediately following irradiation the cultures were transferred to serum-containing medium Time lapse cinemicrography performed during the next 48 h showed a profo ndl d st bed m
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cytoplasmic ramification of the irradiated cells but otherwise no detectable alterations in structure or distribution of either microtubules or microfilaments It is suggested that the alteration in the arrangement of filament bundles is of importance to the impaired locomotion of the irradiated cells

Key words X-irradiation cultured human cells transmission electron microscopy cell motility microtubules microfilaments

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Heavy X irradiation of cultured human non cycling glial cells blocked in G_1 by *density* dependent inhibition of cell division results within 24 h in (a) Alterations in cell shape (b) increased and structurally abnormal plasma membrane motility associated with macropinocytosis (rarely found in dense cultures) and (c) enhanced autophagocytosis with accumulation of autophagic vacuoles containing cell sap and cytoplasmic organelles in various stages of degradation (*Hamberg et al* 1976 1977 1978) The possible relation between these alterations is not clear Increased plasma membrane motility with associated internalization of the plasma membrane may serve to eliminate damaged parts of the membrane or provide membrane material for the formation of autophagic vacuoles within the cytoplasm It may also signify damage to the motility apparatus of the cell or to the regulatory mechanisms thereof

Cell mo ...

... *Porter* 1970) The microtubules seem to be part of a »cytoskeletal« system responsible for the maintenance of the normal shape and polarity of cells (*Porter* 1966 *Gail & Boone* 1971) The microfilaments are believed to participate in many motile processes for example membrane ruffling pinocytosis and cell locomotion (*Wessels et al* 1973)

It appeared interesting to investigate if there were any alterations in the filamentous structures associated with the derangements of cell shape and plasma membrane motility of the irradiated cells We also wished to know if the pattern of locomotion was

disturbed in the irradiated cells since locomotion seems to be closely connected with plasma membrane motility and cell shape

In order to study the alterations of cellular motility and locomotion in some detail it is necessary to use cultures where the cells are sparsely grown and thus not in a state of density-dependent inhibition of growth and locomotion In order to obtain sparse cultures of cells in interphase we subjected the cultures to serum depletion which is known to result in ceased DNA synthesis and arrest in G_1 within 24 h (*Lindgren et al* 1975 1976)

A rewarding method for ultrastructural evaluation of the three-dimensional organization of cultured cells is the »whole cell technique« where cells are cultured on carbon/formvar-coated grids and studied by TEM after fixation and critical point drying Another approach uses fluoresceinlabelled antibodies to tubulin and actin

MATERIAL AND METHODS

Culture Conditions and Irradiation Procedure

The experiments were performed on *in vitro* cultured human glial cells of the U 787 CG line established in culture by *B Westermark* The Wallenberg Laboratory Uppsala according to the methods described by *Pontén and MacIntyre* (1968) Only phase II cells in early passages were utilized and culture conditions were as described earlier (*Hamberg et al* 1976)

A cell suspension consisting of 20 000 cells/ml in Eagle s MEM (EMEM) fortified with 10% calf serum was seeded in 36 mm plastic Petri dishes 2 5 ml in each dish For time lapse cinemicrography and immunofluorescence microscopy the dishes contained round (25 mm) or square (18 mm) glass coverslips respectively For SEM the cells were grown on 12 × 6 × 1 mm glass slides and for TEM on carbon/formvar coated gold grids (*Gershenbaum et al* 1974 *Wolosewich and Porter* 1976 *Collins et al* 1978)

Twenty four hours after seeding the EMEM was replaced by Ham s F10 medium with 0 1% human serum albumin (*Brunk et al* 1977) After another 24 h the cells were exposed to 8 MV X irradiation 200 Gy at a dose rate of 17 Gy/min (*Hamberg et al* 1976) Immediately following irradiation the serum deficient medium was replaced by F10 medium supplemented with 10% calf serum

Time Lapse Cinemicrography and Phase Contrast Light Microscopy

Time lapse cinemicrography covered the interval between 1 and 48 h after irradiation The filming was performed in essentially the same way as described earlier (*Hamberg et al* 1978) although the film speed was kept low (3–10 exposures/min in the various experiments) in order to facilitate surveillance of cell movements during prolonged uninterrupted periods

For phase contrast microscopy cells were fixed in [illegible] glutaraldehyde in 0 1 M Na-cacodylate HCl buffer [illegible] 0 1 M sucrose (pH 7 2 total osmolality ~ 510 mOsm [illegible] vehicle osmolality ~ 300 mOsmol) (*Collins et al* 19[illegible] at 6 12 18 24 and 48 h after irradiation and prep[illegible] as described earlier (*Hamberg et al* 1978)

Electron Microscopy

For scanning electron microscopy cells were fix[illegible] the glutaraldehyde fixative mentioned above postfix[illegible] 1% OsO_4 in 0 15 M Na-cacodylate buffer for 90 [illegible] and critical point dried from CO_2 as previously des[illegible] bed (*Hamberg et al* 1978) For transmission elec[illegible] microscopy cells growing on gold grids were harv[illegible] 24 and 48 h after irradiation glutaraldehyde-OsO_4 f[illegible] and critical point dried as for SEM (*Collins et al* 19[illegible] The grids with cells were studied in a Jeol 10[illegible] microscope with a side entrance goniometer at 100[illegible] Stereo pair micrographs were taken at various angle[illegible] to 20° depending on the magnification

Immunofluorescence Microscopy

The cultures were fixed at the same intervals a[illegible] TEM i e 24 and 48 h after irradiation The ind[illegible] immunofluorescent technique for the staining of [illegible] plasmic microtubules is well established (*Weber e*[illegible] 1975 1978) The cells were fixed in prewarmed (3[illegible] 2% formaldehyde in PBS The plasma membranes [illegible] made permeable to macromolecules by incubation [illegible] min in 0 1% Triton X 100 at room temperature [illegible] cells were then incubated with affinity purified [illegible] antibodies to tubulin and later with fluorescein iso[illegible] cyanate conjugated swine anti rabbit IgG Betwee[illegible] aforementioned steps the coverslips were washe[illegible] PBS and finally mounted cell side down in Elvar[illegible]

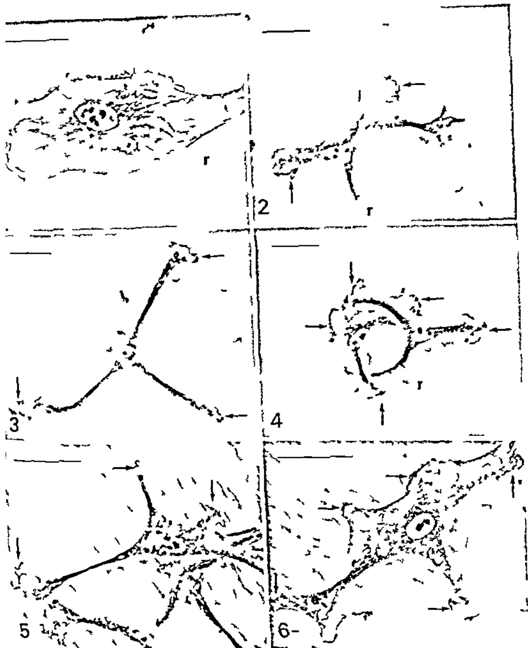
A technique taking advantage of the affinity of D[illegible] I for actin was utilized for the visualization of [illegible] filaments (*Wang and Goldberg* 1976 *Lindberg e*[illegible] 1979 *Carlsson* 1979) Following fixation and treat[illegible] with Triton X 100 the cells were incubated in a sol[illegible] of electrophoretically purified DNase I then [illegible] antibodies to DNase I and finally FITC conjugated s[illegible] anti rabbit IgG

RESULTS

Phase Contrast Microscopy Time Lapse Cin[illegible]crography and SEM

Control cells After 24 h in serum free med[illegible] the cultures consisted mainly of elongated bipol[illegible] stellate cells often with a perinuclear cluste[illegible] vacuoles Ruffles were few and small in size [illegible] picture was consistent with earlier reports on [illegible] response of this glia cell line to serum depriva[illegible] (*Schellens et al* 1976 *Brunk et al* 1977)

Following the change to a medium contai[illegible] 10% calf serum the perinuclear vacuoles grad[illegible] disappeared during the first 12 hours concomita[illegible] with the appearance of more numerous and la[illegible]



Figs 1–6 Sparsely grow ng cells f xed n glutaraldehyde 24 h after return from serum free to serum-conta ning g owth med um Phase contrast l ght m c oscopy Magnif cation nd ca ed by bars representing 50µm

Fig 1 A control cell w th a typ cal lead ng edge w th ruffl ng lame as (*a row*) Note regular cell shape and retract on f b s a the tra ng end of he ce l (*r*)

Figs 2–6 I radia ed cells w th va ab e regular conf gurations and mu tip e lamellas w th ruffles (*a rows*) Most ruffl ng a eas a e sma er than normal and sometimes tuf l ke (F g 6) Macrop nocytotic vacuoles are somet mes fo med as best seen n F g 5 Re raction f br ls are nd cated (*r*)

disturbed in the irradiated cells, since locomotion seems to be closely connected with plasma membrane motility and cell shape

In order to study the alterations of cellular motility and locomotion in some detail, it is necessary to use cultures where the cells are sparsely grown and thus not in a state of density-dependent inhibition of growth and locomotion In order to obtain sparse cultures of cells in interphase we subjected the cultures to serum depletion, which is known to result in ceased DNA synthesis and arrest in G_1 within 24 h (*Lindgren et al* 1975, 1976)

A rewarding method for ultrastructural evaluation of the three dimensional organization of cultured cells is the »whole cell technique«, where cells are cultured on carbon/formvar-coated grids and studied by TEM after fixation and critical point drying Another approach uses fluoresceinlabelled antibodies to tubulin and actin

MATERIAL AND METHODS

Culture Conditions and Irradiation Procedure

The experiments were performed on *in vitro* cultured human glial cells of the U 787 CG line established in culture by *B Westermark* The Wallenberg Laboratory Uppsala according to the methods described by *Ponten and MacIntyre* (1968) Only phase II cells in early passages were utilized and culture conditions were as described earlier (*Hamberg et al* 1976)

A cell suspension consisting of 20 000 cells/ml in Eagle s MEM (EMEM) fortified with 10% calf serum was seeded in 36 mm plastic Petri dishes 2 5 ml in each dish For time lapse cinemicrography and immunofluorescence microscopy the dishes contained round (25 mm) or square (18 mm) glass coverslips respectively For SEM the cells were grown on 12 × 6 × 1 mm glass slides and for TEM on carbon/formvar coated gold grids (*Gershenbaum et al* 1974 *Wolosewich and Porter* 1976 *Collins et al* 1978)

Twenty four hours after seeding the EMEM was replaced by Ham s F10 medium with 0 1% human serum albumin (*Brunk et al* 1977) After another 24 h the cells were exposed to 8 MV X irradiation 200 Gy at a dose rate of 17 Gy/min (*Hamberg et al* 1976) Immediately following irradiation the serum deficient medium was replaced by F10 medium supplemented with 10% calf serum

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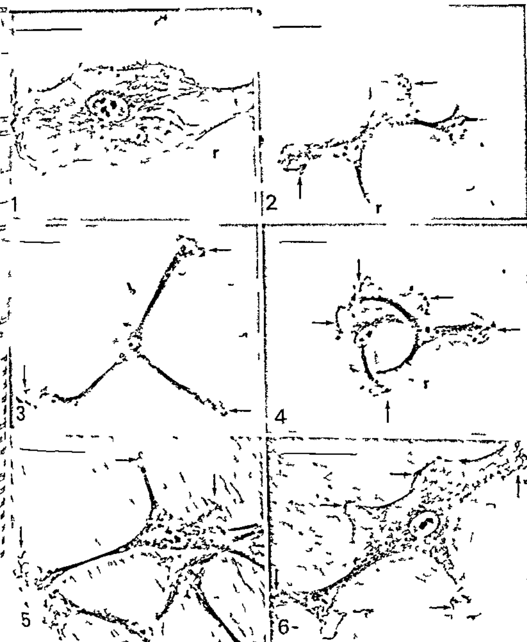
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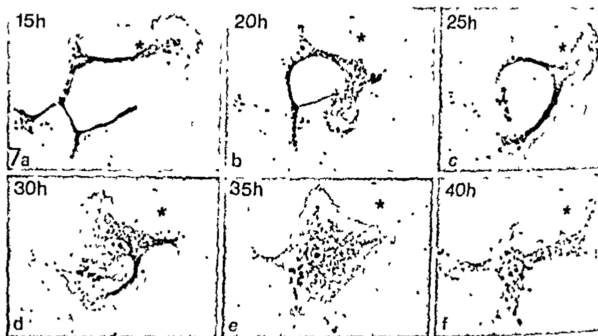


Fig 7 Time-lapse sequence (a–f) showing an irradiated cell changing shape without the formation of a definite lead edge The cell shows almost no net locomotion as seen by comparing the position of the nucleus in relation to indicated fixed point (*) on the coverslip Time in hours after irradiation is indicated in the top left hand corner

ruffles at the cell borders and with diminution of the slender cytoplasmic extrusions At 24 h, and even more so at 48 h, most of the control cells had regained an almost normal cell shape with a well defined leading lamella and a broad, fan-shaped ruffling area with associated intermittent macropinocytosis, and often with retraction fibrils at the end of the cell opposite the leading lamella (Fig 1) Cell locomotion followed a normal pattern with considerable net movement during the observation period (*Pontén et al* 1969)

Irradiated cells During the first 12 h after irradiation and return to a serum-containing medium, the irradiated cells appeared to resume ruffling activity in a manner similar to control cells From that time on however, it became evident that the irradiated cells failed to regain the orderly cell polarization and movement characteristic of controls Instead they became increasingly ramified with broad or slender extrusions and multiple, small, often tuft-like ruffles occurring simultaneously at different parts of the cell border (Figs 2–6)

Best revealed by time-lapse cinemicrography, the irradiated cells sometimes formed one domin broad lamella with a ruffling area, on a par w controls regaining normal polarity and locomoti However, this orderly orientation did not persist the irradiated cells, and the morphology show

of the central cell body with its nucleus (Fig 7)

Whole Cell TEM

Control cells The fine structure of the cont cells 24 and 48 hours after return to seru containing medium did not differ from earl findings regarding proliferating cells of this cell li (*Collins et al* 1978, 1979) Thus the cells of displayed a wide leading lamella with a t lamellopodium forming a broad ruffling area w occasional associated macropinocytotic vacuo (Fig 8) The leading lamella contained microfi ments some of which were arranged in bund oriented in the direction of movement of the c (Fig 10) Microfilament bundles were also se

Fig 8 Peripheral area of control cell growing on carbon/formvar coated gold grid and studied at 100 kV by TE after fixation and CPD 48 h after return to serum-containing medium A ruffling area is depicted No macropinocytotic vacuoles emerging behind the ruffle (*v*) Many slender sometimes branched mitochondria (*m*) a revealed Bar = 5 μm

Fig 9 Electron micrograph of a peripheral part of a cell 48 h after irradiation and change to serum-containi medium Many large lysosomes (*ly*) surround the nucleus (outside the depicted area) Small, tuft like, rudimental ruffles with associated long microspikes are indicated by arrows Bar = 3 μm

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ly

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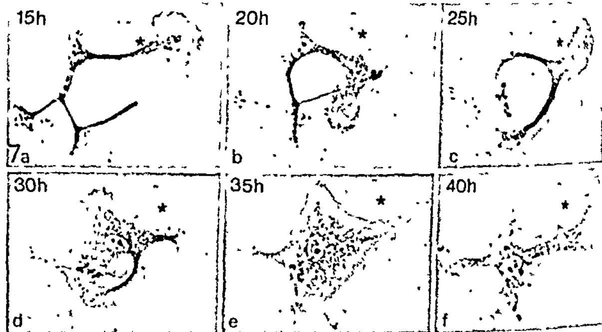


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8

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Fig 10 Control cell 48 h after reversal of serum starvation Part of leading lamella with bundles of microfilaments (*large arrows*) and numerous microtubules (*small arrows*) but no other organelles Bar = 2 µm

Fig 11 Peripheral part of a cell 48 h after irradiation Microfilament bundles (*large arrows*) and microtubules (*small arrows*) are well preserved and show no detectable alterations as compared with the controls There are several membrane veils or small ruffles along the cell border Note the presence of numerous coated vesicles (*cv*) Bar = 3 µm

adjacent and parallel to the cell borders except at the foremost end of the leading lamella and were innumerous in the retraction fibrils found at the trailing end of the cell A great number of microtubules with no discernible pattern of organization were also present in the leading lamella (Fig 10) The lamellipodia contained no organelles or

more numerous in the perinuclear area (not accessible to detailed studies by reason of its thickness) (Fig 8)

Irradiated cells The fine structure of the irradiated cells was difficult to study in the central parts of the cells and the slender extrusions where the cell body seemed to be condensed and impenetrable However the thin flattened lamellar areas at the end of the extrusions or elsewhere on the cell periphery were seen to contain microtubules and microfilaments which in structure and arrangement resembled the observations in the control cells (Figs 9 and 11) The light and scanning electron microscopy findings of small undeveloped and irregular ruffles were conf

ruffles of some malignant glioma cell lines (*Collins et al* 1979) Many small vesicles were demonstrated (Fig 11) and at 48 h after irradiation large vacuolated lysosomes abounded in the central parts of the cells (Fig 9)

Immunofluorescence Microscopy

The pattern of fluorescent antibody stained microtubules in the non irradiated glial cells showed no major divergences from other interphase cells (*Weber* 1976 *Brinkley et al* 1976) The cells contained an elaborate array of abundant straight or curved microtubules which were often arranged in a radiating fashion around the nucleus and terminating close to the cell border

Furthermore the fluorescence pattern obtained with the DNase antibody system resembled that resulting from the use of actin antibodies on other tissue culture cells (*Lazarides* 1975 *Goldman et al* 1975) The fluorescence in the glial cells was largely diffuse and most intense in the perinuclear region with a few thin elongated filaments arranged mainly along the line of movement of the cell A speckled rather intense fluorescence was also observed immediately associated with the ruffling membranes A detailed report on the microfilamentous system in the glial cells will be published elsewhere (*Hoglund et al*)

While the control cells contained filament bundles arranged mainly along the direction of movement of the cells the irradiated ones displayed stainable filaments and microtubules along the longitudinal axes of practically all the asymmetric cellular processes In the flattened lamellar parts of

compared with controls

DISCUSSION

The effects of irradiation on the serum deprived sparsely grown glial cells may not be directly comparable with the results of our previous studies utilizing density-dependent growth inhibited cells because the radiosensitivity may differ However serum depletion appears to be the best method available to block a sparse culture of cells in G_1 during irradiation By so doing the sensitive replication phase is avoided and yet the block can be reverted after irradiation allowing study on any induced disturbance in cell movement and locomotion Furthermore the whole cell TEM and the immunofluorescent techniques do not lend themselves to observation of densely grown and partly overlapping cells

Following stimulation with calf serum the irradiated cells started ruffling of the peripheral parts of the plasma membrane in much the same way as the controls No definite polarization and coordinated locomotion ensued however Instead ruffling was distributed in small areas all round the cell periphery and peripheral lamellar areas at


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different ends of the cells became dominant one after the other, but *only briefly and* without resultant significant net movement of the cells This disturbed locomotor activity resembles the effects of *colchicine/colcemid* on cultured cells (*Goldman* 1971, *Gail and Boone* 1971) but in contrast to colchicine treated cells the irradiated glial cells retained their asymmetry The studies on phagokinetic tracks of *3T3* cells by *Albrecht-Buehler* (1977) has revealed that the main actin-*containing bundles* parallel the direction of migration of cultured cells on their substratum *In the irradiated* glial cells filament bundles projected into several cytoplasmic *extrusions* mostly without any dominating direction It seems possible that this alteration in the arrangement of filament bundles is of importance to the impaired locomotion of the irradiated cells The peripheral lamellar parts of the irradiated cells displayed no obvious alterations in the structure or distribution of neither microtubules nor microfilaments However, the possibility of damage to microtubules and/or microfilaments *at a level not* perceptible by the methods used remains Furthermore irradiation may have an adverse effect on the *regulatory systems of cell* movement and locomotion The possible implications of the altered cell motility *on plasma* membrane turnover remain obscure

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Immunofluorescence stainings were performed by *R Karlsson* the CEMO group the Wallenberg Laboratory University of Uppsala Uppsala Sweden

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AUTOLOGOUS IMMUNE COMPLEX NEPHRITIS AND DOCA-NaCl LOAD A NEW MODEL OF HYPERTENSION

ILKKA TIKKANEN FREJ FYHRQUIST AARO MIETTINEN and TOM TÖRNROTH

The Minerva Institute for Medical Research Department of Bacteriology and Immunology University of Helsinki and IVth Department of Medicine Helsinki University Central Hospital Helsinki Finland

Tikkanen I Fyhrquist F Miettinen A & Tornroth T Autologous immune complex nephritis and DOCA NaCl load a new model of hypertension Acta path microbiol scand Sect. A 88 241–250 1980

In order to explore immunological features of hypertension we studied autologous immune complex nephritis (Heymann nephritis) combined with DOCA NaCl treatment This combination resulted in hypertension and increased heart weight whereas DOCA NaCl treatment alone induced only a slight elevation of blood pressure and a moderate increase in heart weight Nephritic rats without DOCA NaCl load remained normotensive their heart weights being comparable to those of controls This new model of hypertension was neither characterized by azotemia nor by reduced renal excretory capacity Hypertension was not renin angiotensin-dependent DOCA NaCl treatment accelerated the development of proteinuria In the hypertensive rats systolic blood pressure correlated to daily urinary protein excretion Renal histopathology revealed changes resembling those of malignant nephrosclerosis Immunohistology and electron microscopy showed a typical membranous glomerulonephritis in all immunized animals It was concluded that immune complex disease of the Heymann nephritis type may interfere with normal hemodynamic adaptation to hypervolemic sodium load resulting in hypertension

Key words Hypertension nephritis DOCA NaCl immune complexes rat

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The involvement of immunological mechanisms in hypertension has been suggested by several authors (for references see 22 25) In experimental animals gamma globulin deposits have been found in vascular lesions caused by deoxycorticosterone NaCl (DOCA NaCl) hypertension (30) and hyper tension following partial renal infarction (Loomis type) in which autoantibodies against arterial wall and kidney tissue were detected as well (37) Hypertensive arterial damage is accompanied by an inflammatory cellular reaction regardless of the experimental model used (7 33) This reaction seems to be thymus-dependent in DOCA NaCl and mice (33) The thymus was not however important for the development and early phases of hypertension

The severity of lesions in serum sickness is increased by hypertension (38) Hypertension may be associated with the development of arteritis (7, 28) On the other hand NZB mice with spontane ous immune complex disease get hypertension which can be ameliorated by immunosuppression (32) We therefore studied the effect of a chronic immune complex disease on the development of hypertension Autologous immune complex glome

treatment in female Wistar rats

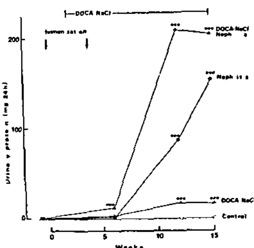


Fig 2 Onset and severity of proteinuria in different experimental groups * p<0 05 ** p<0 01 *** p<0 001 compared with controls

NaCl load remained normotensive throughout the study Heart weights parallelled systolic blood pressure and were most markedly increased in the DOCA NaCl nephritis group moderately increased in DOCA NaCl rats and corresponded to that on controls in the nephritis group (Table 1)

Proteinuria and serum proteins Proteinuria developed earlier in the nephritic rats treated with DOCA NaCl than in the non treated ones (p<0 01 at 6 weeks Fig 2) A significant correlation was found between systolic pressure and degree of proteinuria in the DOCA NaCl nephritis group at the end of the experiment (r = 0 82, p<0 01) Serum protein was slightly decreased in the DOCA-NaCl nephritis group (Table 1) α-Globulins increased slightly while β- and γ globulins decreased in both nephritic groups Serum albumin concentration did not decrease Ascites or edema were not observed

Creatinine clearance and renal handling of electrolytes None of the immunized rats became azotemic, whether treated with DOCA NaCl or not (Table 1) No differences were observed in weight gain urine volumes urinary sodium and potassium excretion and the sodium/potassium ratio (Table 2) and serum sodium and potassium concentrations (Table 1) between the hypertensive DOCA NaCl-nephritis and the non hypertensive DOCA NaCl groups Both increased their urine output 6 fold urinary sodium/potassium ratio 10 fold and sodium excretion up to 20 fold compared with the control animals

Plasma renin activity and serum osmolality (Table 1) PRA was clearly suppressed in both gorups treated with DOCA NaCl Serum osmolality values did not differ

Histological findings All immunized animals had membranous glomerulonephritis visible in PASM-stained paraffin sections as either/ both a diffuse vacualization (36) or/and diffuse projections of the glomerular basement membrane (Table 3) Proliferative sclerotic glomerular changes were present in the DOCA NaCl nephritis group, resembling the picture of malignant nephrosclerosis (Fig 3)

TABLE 1 *Heart Weight and Analytical Findings at the End of Experiment (Mean ± SD)*

		Control (n = 11)	Nephritis (n = 11)	DOCA NaCl (n = 11)	DOCA NaCl nephritis (n = 10)
Heart weight/body weight (mg/g)		3 03 ± 0 23	3 15 ± 0 23	3 65 ± 0 22***	3 98 ± 0 29++ *
Hematocrit (%)		47 4 ± 2 4	46 1 ± 2 7	46 1 ± 2 9	44 7 ± 3 8*
Serum proteins (g/l)	total	70 5 ± 1 7	68 8 ± 3 9	68 4 ± 3 0	63 5 ± 10 3*
	albumin	44 2 ± 1 9	48 3 ± 3 9**	45 1 ± 2 7	44 9 ± 7 0
	α globulin	5 1 ± 1 0	6 8 ± 1 7**	5 7 ± 1 2	7 6 ± 2 8*
	β globulin	12 6 ± 2 8	9 1 ± 2 7**	11 3 ± 1 9	7 7 ± 3 1++
	γ globulin	7 6 ± 1 1	4 1 ± 1 9***	6 0 ± 1 6	3 6 ± 2 4+ **
Serum sodium (mmol/l)		143 ± 2 9	141 ± 3 9	145 ± 0 9*	144 ± 5 1
Serum potassium (mmol/l)		5 0 ± 0 4	5 2 ± 0 6	4 0 ± 0 5***	4 2 ± 0 3***
Creatinine clearance (ml/min)		0 95 ± 0 30	1 17 ± 0 24	1 69 ± 0 30***	1 49 ± 0 26***
Plasma renin activity (ng/ml/h)		14 2 ± 6 9	11 2 ± 8 6	0 04 ± 0 12***	0***†
Serum osmolality (mosmol/kg)		297 ± 11	294 ± 14	291 ± 15	285 ± 16

* p<0 05 ** p<0 01 *** p<0 001 compared with controls
\+ p<0 05 + + p<0 01 DOCA NaCl nephritis compared with DOCA NaCl
† below limit of assay sensitivity

MATERIAL AND METHODS

General design 44 female Wistar rats (Animark Helsinki Finland) weighing about 180 g were divided into 4 equal groups 1) *Control* animals received only sham injections and immunizations 2) *Nephritis* group immunized (see below) to induce autologous immune complex nephritis 3) *DOCA-NaCl* group received weekly DOCA (Percorten M Ciba 30 mg/kg s c) injections and 0 9% NaCl solution as the only drink 4) *DOCA-NaCl nephritis* group with a combination of DOCA NaCl treatment and nephritis

DOCA NaCl regimen was started 2 weeks after the first immunization At 16 weeks blood was collected from abdominal aorta under ether anesthesia Hematocrit was determined EDTA plasma 6 g of Na_2EDTA/liter of blood and serum were separated by centrifugation and stored at −20 °C until assayed

Induction of autologous immune complex nephritis A brush border (BB) membrane fraction was isolated from kidney cortices of Sprague Dawley rats as described (26) Each rat received 2 injections of BB antigen mixed with equal amounts of Freund s complete adjuvant (Difco Laboratories Detroit USA) containing 5 mg of Mycobacterium tuberculosis H 37 Ra (Difco) per ml At the first immunization 0 4 ml of antigen emulsion with 200 µg of BB protein was given into foot pads and 25 µl of pertussis vaccine (Per Vaccin Orion Pharmaceuticals Helsinki Finland) containing 22×10^6 killed Hemophilus pertussis bacteria/ ml was injected into the dorsum of each foot (21) 4 weeks later BB antigen emulsion was given subcutaneously without pertussis vaccine This immunization schedule results in the development of autologous immune complex nephritis within 2 months (27)

Blood pressure recordings Systolic blood pressure was measured at regular intervals in conscious animals by a tail cuff (Harvard Apparatus Co Massachusetts USA) method using a Doppler ultrasonic flowmeter (Parks Electronics Lab Beaverton USA) to detect the pulse (6) The tail was first warmed for 10 min at 37 °C

Analytical methods Urinary protein output was measured from 18 h samples collected individually by the method of Lowry et al (24) after trichloracetic acid precipitation Total serum proteins were determined by the Biuret method analyzed electrophoretically on cellulose acetate membranes and the concentrations of albumin α β and γ globulins were calculated densitometrically (Helena autoscanner Helena Lab Beaumont

[illegible]

concentrations were measured by a modification (19) of the kinetic creatinine assay (Merckotest 3384 Merck) Serum osmolality was determined by a vapor pressure osmometer (Wescor Inc Logan USA) Plasma renin activity (PRA) was determined by radioimmunoassay (11) modified for rat plasma using 5 0 mM hydroxyquinoline as enzyme inhibitor and pH 6 5 during the [illegible]

[illegible] ions were [illegible]bedded in paraffin Sections 2–3 µ thick were stained with hematoxylin and eosin (H&E) periodic acid Schiff (PAS) periodic acid silver methenamine (PASM) and Masson s trichrome

Immunofluorescence (IFL) studies 5 µ cryostat sections of the snap frozen kidney and heart samples were fixed for 10 min in −20 °C acetone and washed in phosphate buffered saline (PBS) pH 7 2 For direct IFL cryostat sections were reacted with fluorescein isothiocyanate (FITC)-conjugated antisera for 30 min washed in PBS for 10 min and mounted in buffered glycerol Serum BB autoantibodies were determined by an indirect IFL technique For this 5 µ cryostat sections of normal rat kidney were reacted with rat sera diluted $^1/_2$ and $^1/_4$ in PBS for 30 min washed in PBS for 10 min reacted with FITC conjugated anti rat IgG for 30 min and after a final wash mounted in glycerol The stained sections were studied with a Zeiss standard microscope (23) The F/p ratios of the FITC conjugated rabbit anti rat IgG β_1C/C_3 fibrinogen and albumin (Nordic Immunological Lab Tilburg The Netherlands) were 2 3 1 7 3 [illegible] and 1 5 and the protein contents of the dilutions used were 0 5 mg/ml 2 3 mg/ml 0 7 mg/ml and 0 4 mg/ml respectively

Electron microscopy Specimens from 5 rats (one from group 2 and 2 from groups 2 and 4) were processed for electron microscopic study (35) Two glomeruli from each of the 5 rats were examined

Statistics The significance of the differences between mean values was evaluated by Student s t test preceded by logarithmic transformation when values showed skewed distribution

RESULTS

Blood pressure Only rats with both nephritis and DOCA-NaCl treatment became hypertensive (Fig 1) DOCA NaCl alone induced only a slight increase in blood pressure Nephritic rats without DOCA

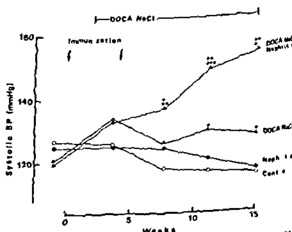


Fig 1 Systolic blood pressure during the experiment * p<0 05 ** p<0 01 *** p<0 001 compared with control + p<0 05 ++ p<0 01 DOCA NaCl nephritis compared with DOCA NaCl

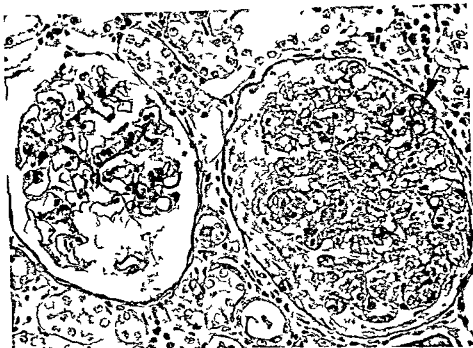


Fig 3 An almost normal glomerulus and a glomerulus with extensive proliferative sclerotic changes including hyaline subendothelial or intraluminal deposits (arrow) From DOCA NaCl nephritis group PAS stain × 310

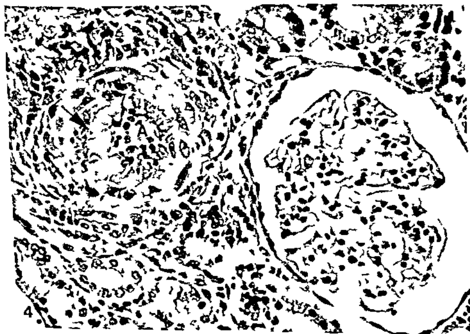


Fig 4 An intralobular artery (A) with marked thickening fibrinoid necrosis (arrow) and inflammatory cell infiltration of the wall The glomerulus appears normal at this magnification From DOCA NaCl nephritis group H&E stain, × 320

TABLE 2 *Body Weight and Urine Electrolytes during the Experiment (Mean ± SD)*

	Time (weeks)	Control (n = 11)	Nephritis (n = 11)	DOCA-NaCl (n = 11)	DOCA NaCl nephritis (n = 11)
Body weight (g)	0	236 ± 8 2	237 ± 13	241 ± 7 8	238 ± 11
	6	289 ± 11	276 ± 14*	287 ± 17	288 ± 18
	12	293 ± 8 7	293 ± 14	303 ± 16	302 ± 18
	15	303 ± 12	298 ± 18	308 ± 18	307 ± 21
Urine volumes (ml/24 h)	0	6 6 ± 2 6	5 2 ± 2 9	7 4 ± 4 2	6 3 ± 3 6
	6	10 1 ± 4 8	10 8 ± 5 7	27 5 ± 13 ***	27 9 ± 7 8***
	12	9 3 ± 4 8	10 4 ± 4 4	50 9 ± 28 ***	45 3 ± 13***
	15	10 5 ± 9 0	14 3 ± 10	63 9 ± 28 ***	64 8 ± 22 ***†
Sodium excretion (mmol/24 h)	0	0 34 ± 0 10	0 30 ± 0 14	0 43 ± 0 19	0 46 ± 0 21
	6	0 47 ± 0 17	0 43 ± 0 21	3 69 ± 2 0***	3 75 ± 1 1***
	12	0 36 ± 0 15	0 39 ± 0 12	8 56 ± 5 0***	6 91 ± 1 9***
	15	0 42 ± 0 20	0 27 ± 0 10*	8 63 ± 3 4***	9 27 ± 3 9***†
Potassium excretion (mmol/24 h)	0	0 89 ± 0 24	0 75 ± 0 22	0 79 ± 0 27	0 91 ± 0 21
	6	0 79 ± 0 16	0 82 ± 0 19	0 77 ± 0 20	0 72 ± 0 20
	12	0 63 ± 0 21	0 72 ± 0 24	1 21 ± 0 50**	1 28 ± 0 42***
	15	0 57 ± 0 31	0 61 ± 0 16	0 98 ± 0 29**	1 28 ± 0 59**†
Urine sodium/potassium	0	0 38 ± 0 18	0 45 ± 0 24	0 59 ± 0 25	0 49 ± 0 13
	6	0 58 ± 0 14	0 55 ± 0 30	4 63 ± 1 7***	5 29 ± 1 9***
	12	0 63 ± 0 32	0 57 ± 0 22	6 76 ± 1 7***	5 73 ± 1 8***
	15	0 83 ± 0 32	0 47 ± 0 17**	8 75 ± 2 8***	7 82 ± 3 0***†

* $p<0\ 05$ ** $p<0\ 01$, *** $p<0\ 001$ compared with controls, † n = 10
DOCA-NaCl-nephritis compared with DOCA-NaCl all differences non significant

However, only 2 rats had vascular changes, which also affected arteries larger than arterioles (Fig 4) One rat in the DOCA-NaCl group had similar but milder glomerulosclerotic changes It is noteworthy that all rats with these proliferative-sclerotic lesions had both heavy proteinuria and high blood pressure Tubulo-interstitial changes, mostly degenerative and inflammatory seemed to reflect the severity of the glomerular disease

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(Fig 5) C_3 deposited in a similar way was u- te in 20% of the rats Granular, often focal an segmental glomerular deposits of albumin wer frequently seen Tubular cytoplasmic and lumina albumin, IgG and C_3 were seen in most immunize animals No systematic differences were see

TABLE 3 *Histopathological Changes in the Kidneys*

	Control (n = 11)	Nephritis (n = 11)	DOCA NaCl (n = 11)	DOCA-NaCl nephritis (n = 11)
Membranous glomerulonephritis	–	11	–	11
Proliferative sclerotic glomerular changes	–	–	1	5
Tubulo intersitial changes	–	2	1	6
Vascular changes	–	–	–	2



Fig 3 An almost normal glomerulus and a glomerulus with extensive proliferative sclerotic changes including hyaline subendothelial or intraluminal deposits (arrow) From DOCA NaCl nephritis group PAS stain × 310

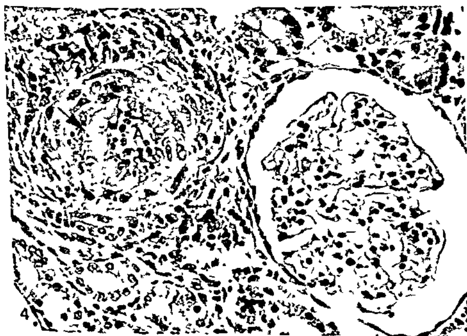
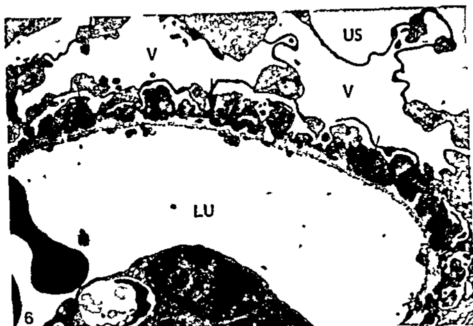


Fig 4 An intralobular artery (A) with marked thickening fibrinoid necrosis (arrow) and inflammatory cell infiltration of the wall The glomerulus appears normal at this magnification From DOCA NaCl nephritis group H&E stain, × 320

Fig 5 Immunofluorescence micrograph showing granular deposits of IgG along glomerular capillary walls kidney of a rat immunized with the brush border antigen and treated with DOCA NaCl Staining with F conjugated anti rat IgG × 1300

TABLE 4 *Immunohistological Findings in the Kidneys and Serum Brush Border (BB) Autoantibodies at the En Experiment*

		Control (n = 11)	Nephritis (n = 11)	DOCA NaCl (n = 11)	DOCA NaC nephritis (n = 10)
Glomeruli					
Granular deposits of IgG along the capillary walls		–	11	–	10
Granular deposits of C_3 along the capillary walls		–	2	–	2
Tubuli					
IgG	Lumen/BB	–	7	–	6
	Cytoplasm	–	6	–	5
C_3	Lumen/BB	–	8	1	6
	Cytoplasm	–	4	–	2
Fibrinogen	Lumen/BB	–	5	–	7
	Cytoplasm	–	2	–	2
Albumin	Lumen/BB	–	4	–	3
	Cytoplasm	–	7	2	6
Serum					
Brush border antibodies		–	10	–	8



g 6 Electron micrograph of glomerular capillary showing multiple subepithelial electron-dense deposits (arrows) iaracteristic of membranous glomerulonephritis The DOCA NaCl nephritis group V epithel al vesicles .U cap llary lumen US – urinary space × 6000

etween nephritic rats treated or untreated with DOCA NaCl In non immunized rats only irregu ar mainly mesangial deposits of IgG were seen Ne found no deposits of IgG or C_3 in the arterioles of the kidneys of any rat At the end of the experiment brush border autoantibodies were present in the sera of 18 of the 21 immunized rats (Table 4)

Electron microscopic stud es The four rats from groups 2 and 4 showed multiple subepithelial or intramembranous electron dense deposits corre spond ng to phase B and phase C membranous glomerulonephritis (36) (Fig 6) No ultrastructural d fferences were noted between the two groups In add tion these rats showed huge blebs or vesicles within the cytoplasm of glomerular visceral epithelial cells (Fig 6) The significance of these vesicles which have been descibed previously in N N'-diacetyl benzidine induced nephrosis (9) is as yet unknown

DISCUSSION

Hypertension developed only in rats with both immune complex nephritis and DOCA NaCl load in the present study This type of hypertension was could it be shown to be due to reduced sodium excretory capacity of kidneys

Heymann nephritis is considered a model of autologous immune complex nephritis (1 15 29) During its development antibodies are formed against renal tubular brush border antigens Circu lating immune complexes are then deposited in the glomeruli A local formation of immune complexes has been suggested in the heterologous type of immune complex nephritis (31) which may also apply to Heymann nephritis Our findings of serum antibodies against renal tubular brush border granular pattern of IgG deposits along basement membranes of glomerular capillaries and subepithe lial localization of electron-dense deposits confirm the observations of others (3 16)

DOCA NaCl treatment results in sodium and water retention increased extracellular and plasma volumes increased cardiac output and hypertension (8 13) which may later predominantly be due to elevated peripheral resistance but show normalized cardiac output (8) Susceptibility to DOCA NaCl hypertension varies genetically and in some rat strains sensitization through the removal of one kidney is required in order for hypertension to develop (18 34) With both kidneys left the Wistar rats used in our study were able to adapt to DOCA NaCl treatment without any marked increase in blood pressure (Fig 1)

Hypertension in the DOCA-NaCl-nephritis group would not appear to be renin-angiotensin dependent (Table 1) The hypertensive mechanisms are probably related either to DOCA-induced sodium retention, mechanisms associated with immune complex nephritis or both

According to *Guyton* (17), a given filtration pressure is required in order for a sodium load to be excreted, in hypertension the higher blood pressure actually reflects – for unknown reasons – a resetting of this relationship This 'pressure diuresis' hypothesis as a cause of hypertension is, however, based on work with isolated kidneys and has not been unequivocally demonstrated in the whole organism On the other hand, the capacity of single nephrons to increase their fractional electrolyte excretion compensatorily to nephron loss has been pointed out (5) and also demonstrated in Heymann nephritis (2) In our study, hypertensive DOCA-NaCl-nephritis rats were able to excrete the same amount of sodium and water as non-hypertensive DOCA-NaCl rats throughout the experiment

In nephrotic syndrome associated with Heymann nephritis, inability of sodium excretion has been reported (4) However, such nephrotic syndrome did not develope in the present hypertensive model, since ascites edema and hypoalbuminemia were lacking

Salt-resistant rat strains are able to adapt to increased cardiac output by compensatory peripheral vasodilation (34) On the other hand salt-sensitive rats react by autoregulatory vasoconstriction resulting in elevated peripheral resistance and hypertension The non-hypertensive DOCA NaCl group in our study probably adapted to increased cardiac output by lowering the peripheral resistance In the DOCA NaCl nephritis group elevated blood pressure thus means that peripheral resistance has either remained normal or else been incresed further

DOCA-NaCl treatment accelerated protein leakage from glomerular capillaries (Fig 2) Arterial wall permeability to plasma macromolecules has been shown to be increased in hypertension (14) Gamma globulin deposits have been found in vascular lesions of DOCA NaCl-induced hypertension (30) Hypervolemia may thus promote vascular insudation of immune complexes which may make the peripheral resistance rise and render nephritic rats sensitive to DOCA-NaCl treatment

It is unclear whether proliferative sclerotic glomerular changes in the hypertensive rats are a cause or effect of hypertension Primary and secondary nephroscleroses are usually histologically similar (7) High blood pressure as such may cause nephrosclerosis and, on the other hand sclerotic lesions may be primary, later followed by hypertension *Byrom* (7) has suggested that a weakened vascular wall is susceptible to the mechanical stress of even normal blood pressure, whereas high blood pressure may damage intact vasculature as well Thus the immunological model studied may have accelerated the development of the sclerotic lesions

Giese (14) has shown that during experimentally induced angiotensin hypertension the vascular lesions appear in the areas of dilated arterioles and that increased permeability to plasma components and possibly ischemia of the vascular wall are important in this context The lesions of experimental serum sickness are aggravated by high blood pressure (38) This may explain why immune complex nephritis together with DOCA NaCl load exceeds the glomerular and vascular tolerance resulting in irreversible injury Perturbation of vascular wall metabolism and function takes place in DOCA-NaCl hypertension even prior to the appearance of lesions visible in a light microscope (12)

The data on the new experimental rat model presented here accord with and extend previous data that immunological factors may be involved in the pathogenesis of hypertension (30 32 33 37) This may have important bearings on immunological features in human hypertension as well (22 25)

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THE RELIABILITY OF MALIGNANT THYROID TUMOR DIAGNOSIS IN THE SWEDISH CANCER REGISTRY

Review of 200 Cases

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Holm L E Lowhagen T & Silfversward C The reliability of malignant thyroid tumor diagnosis in the Swedish Cancer Registry Review of 200 cases Acta path microbiol scand Sect A 88 251–254 1980

Two age stratified periodical samples of 100 cases of malignant thyroid tumors from the Stockholm region and 100 such cases from other parts of Sweden reported to the Swedish Cancer Registry between 1958 and 1972 were studied The cytologic and/or histologic slides were re-examined by two pathologists independently In 85 per cent of the cases reported from Stockholm and in 94 per cent of the cases from other parts of Sweden the pathologists agreed on the original diagnosis of malignant tumor The remaining cases (weighted mean 8%) were at re-examination considered to be benign thyroid lesions

Key words Malignant thyroid tumor diagnosis Swedish Cancer Registry

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The histopathologic classification of thyroid tumors has progressed considerably during recent years The most significant publication in this respect is the World Health Organization (WHO) classification (1974) partly based on earlier comprehensive efforts to standardize the diagnostic criteria of these tumors (*Woolner et al* 1961)

Uniformity in the classification of malignant tumors is a prerequisite for epidemiologic studies and for comparison between different medical centers The absence of specific diagnostic criteria prior to the introduction of the WHO classification implies possible variations in the incidence of malignant thyroid tumors reported to different tumor registries In one study 696 cases of such tumors reported in the course of a few years to the national cancer registries of Finland Iceland Norway and Sweden the review showed 12 5 per cent to be benign (*Saxén et al* 1978) It was therefore considered of value to review a representative material of malignant thyroid tumors reported to the Swedish Cancer Registry over a longer period of time and to apply the criteria proposed by WHO to ascertain the reliability of the diagnosis of such tumors in that registry

MATERIALS AND METHODS

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[illegible] region and 100 from other parts of Sweden The distribution of the cases by the original histologic classification is shown in Table 1 Of the reported tumors 100 were originally classified as papillary carcinomas 58 as follicular 16 as anaplastic and 26 as other types of malignant thyroid tumors In

TABLE 1 *Tumor Classification of the Statistical Samples of Malignant Thyroid Tumors Reported to the Swed. Cancer Registry*

Original histologic classification	No of reported carcinomas		
	Stockholm region	Rest of Sweden	Total
Papillary carcinoma	49	51	100
Follicular carcinoma	30	28	58
Anaplastic carcinoma	11	5	16
Other malignant thyroid tumors	10	16	26
Total	100	100	200

this last group were included 4 medullary carcinomas and also cases with unspecific diagnoses, such as adenocarcinoma and carcinoma

Information on the name and date of birth of the patients was obtained from the Swedish Cancer Registry The histologic and/or cytologic slides, on which the diagnoses were based, and relevant patient record data from the time of the tumor diagnosis were obtained from the reporting hospitals Available slides were re-examined and re classified on the basis of the WHO classification (1974) by two pathologists (*T Lowhagen and C Silfverswärd*) independently and without knowledge of the original tumor classification The diagnosis was based on histologic slides in 184 cases and on cytologic slides in 16 cases The size of the tumors was estimated from the histologic sections as being less than 15 mm in diameter, 15 mm or more or not known

The tumors were first evaluated as benign or malignant If considered malignant they were further classified into papillary follicular or anaplastic carcinomas or other types of malignant thyroid tumors In cases (n = 21) where the pathologists disagreed the slides were re-examined and re-classified by them simultaneously through a double headed microscope A common decision on the diagnosis and of the classification was reached in all but 2 cases In one of these 2 cases the pathologists disagreed on the diagnosis of malignant tumor and in the other case they differed in the classification In both cases one of them agreed on the original diagnosis and classification these being taken as final

RESULTS

The original diagnosis of malignant thyroid tu was at re-examination confirmed in 85 per cen the cases from the Stockholm region and in 94 cent of the cases from the other parts of Swede

The remaining 21 cases were at review c sidered to be benign lesions, 16 were origin classified as follicular, 3 as papillary and 2 unclassified carcinomas Five of these follic carcinomas and 1 of the papillary carcinomas w reported from outside Stockholm, the remainde the benign lesions being reported from the Sto holm region

The size of the thyroid tumors is shown in T 2 There was no difference in the tumor distribution between the two samples The majo of the 200 cases (64 per cent) had a tumor size o mm or more Nine of the confirmed papil carcinomas were less than 5 mm in diameter size of the lesions considered to be benign at examination was in 10 cases less than 15 mm, u cases 15 mm or more and in 1 case the size was known

The correlation between the original histopa logic diagnoses and those obtained at re-exam tion is presented in Table 3 Agreement obtained in 93 per cent of the cases of papillary

TABLE 2 *Estimated Tumor Size Distributed by the Original Tumor Classification*

Original tumor classification	Tumor size		
	<15 mm No	≥15 mm No	Not known No
Papillary carcinoma	31	61	8
Follicular carcinoma	8	44	6
Anaplastic carcinoma	0	11	5
Other malignant thyroid tumors	3	12	11
Total	42	128	30

TABLE 3 *Statistical Samples of Malignant Thyroid Tumors Distributed by the Original Tumor Classifications and Those Obtained at Re examination*

Original tumor classification	In agreement with original diagnosis No	Other malignant thyroid tumor No	Benign thyroid lesion No
Papillary carcinoma	93	4	3
Follicular carcinoma	36	6	16
Anaplastic carcinoma	12	4	0
Other malignant thyroid tumors	12	12	2
Total	153	26	21

62 per cent of the follicular and in 75 per cent of the anaplastic carcinomas and in 46 per cent of the cases of other types of malignant thyroid tumors

In 9 cases the malignant tumor was diagnosed at autopsy In 2 of the cases considered to be benign at re-examination the original diagnosis of malignant thyro d tumor had already been revised to benign les on The reports to the Swedish Cancer Registry had not been withdrawn however

DISCUSSION

The present study disclosed when using the WHO classification benign thyroid lesions in 15 per cent (95 per cent confidence limits 8 7–23 5) of the cases reported as malignant from the Stockholm region and in 6 per cent (2 2–12 6) of the cases from the rest of Sweden with a weighted mean of 8 per cent (3 5–15 2) These figures are consistent with those found by *Saxen et al* (1978)

The tumours most often erroneously reported as carcinomas were the follicular adenomas A pos s ble explanation for this discrepancy may be that in some of these cases cellular atypia was considered the basis for the diagnosis and in others that areas of hyperplasia in nodular goiter or thyroiditis were misinterpreted as follicular carcinomas At the re examination however the presence of vascular or capsular invasion was the main distinguishing factor between adenomas and carcinomas of the follicular type In a study by *Franssila & Saxen* (1972) the overdiagnosis of atypical nodules and adenomas was considered to be a possible explana tion for the high incidence of follicular carcinomas in areas of endemic goiter

It must be emphasized that this review was based on available slides and the divergence between the diagnoses of the reviewers and the original diagno ses might to some extent be explained by the possibility that all histologic slides without our knowledge were not present at the re-examination.

Furthermore we do not know the proportion of malignant thyroid tumors wrongly registered in the Swedish Cancer Registry as other types of malig nant tumors Two of the 200 malignant thyroid tumors of the present study had at an earlier re examination been revised to benign thyroid lesion but apparently neither of the two reports were withdrawn from the registry

In the samples studied there were 9 carcinomas with a size of less than 5 mm in diameter It is well known that the larger the number of histologic sections of the thyroid gland examined the higher has been the figure obtained for the prevalence of occult carcinoma (*Sampson et al* 1969) Serial sectioning of thyroid glands at autopsy has disclosed carcinomas in 3–28 per cent of the glands examined (*Mortensen et al* 1955 *Sampson et al* 1969 *Fukunaga and Yatani* 1975 *Sasaki et al* 1976 *Sobrinho S moes et al* 1979 [illegible]

[illegible] that genetic and possibly social and cultural factors can be of etiologic importance in explaining this difference It has been suggested too that the majority of occult thyroid carcinomas – which are generally of the papillary type – remain occult and that only a few are detected clinically The fact that the incidence of clinical thyroid carcinoma in the Japanese does not differ significantly from that in many other countries supports this assumption (*Fukunaga and Yatani* 1975)

The regions of Stockholm Malmo and Gothenburg (the three largest cities in Sweden) have an incidence of malignant tumors that is higher than that of the rest of Sweden (*Ringertz* 1971) For malignant thyroid tumors the ratio is 1 4 1 Differences in diagnostic criteria of such tumors can be one factor that partly explains the higher incidence in these regions Whether there is a higher proportion of benign thyroid lesions reported from the regions of Malmo and Gothenburg as was

indicated to be the case for the Stockholm region than for other parts of Sweden is not known There is also a possibility that a higher frequency of thyroid surgery and a higher autopsy rate in these regions might contribute to their higher incidence of malignant thyroid tumors

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We wish to thank Mrs *Elisabeth Bjurstedt* and Mrs *Karin Steen* for their excellent secretarial assistance Mrs *Kerstin Wennerstrom* for helping us find the histologic and cytologic slides of this study and Professor *Gunnar Eklund* for advice in connection with epidemiologic problems

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Acta path. microbiol scand Sect. A 88 255–261 1980

QUANTITATIVE STUDIES OF THE GASTRIN-PRODUCING CELLS OF THE HUMAN ANTRUM

A Methodological Study

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Nielsen H O Halken S & Lorentzen M Quantitative studies of the gastrin producing cells of the human antrum A methodological study Acta path microbiol scand Sect. A 88 255–261 1980

The antral gastrin producing cells (G-cells) have been identified by the indirect immunoperoxidase technique in two antrum preparations removed due to a recurrent duodenal and gastric ulcer Morphometric principles were applied to the G-cells with determination of their volume density numerical density and mean cell volume The study showed that within-observer variation between observer variation and within patient variation were negligible provided at least 200 G-cells were counted A biopsy material can be used as well as larger tissue blocks when this minimum sample size is respected A method for estimating the total G-cell population and the total G-cell volume in the antrum was developed In the antrum removed due to a gastric ulcer the number of G-cells was 190 $\times 10^6$ and their total volume 176 mm^3

Key words Antral gastrin producing cells immunohistochemistry quantitation

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A quantitative estimate of the gastrin producing cells (G-cells) has acquired new interest following the development of selective vagotomy techniques for duodenal and gastric ulcer surgery Unexplained deviations from the expected clinical and physiological responses to this treatment predominantly directed against parietal cell function have raised the question of the role of the G-cell population with regard to the postoperative results This is especially true with regard to the striking difference in recurrence rates after proximal gastric vagotomy for duodenal and gastric ulcer (2)

Different kinds of G-cell abnormality may exist The possibility of a G-cell hyperplasia in some duodenal ulcer patients (15) or a G-cell hypertrophy (7) has been suggested Differences in G-cell distribution between patients with duodenal and gastric ulcer were found by Stave & Brandtzaeg (21) but they observed no relation between the total G-cell population and certain gastro-duodenal diseases At the present time no definite answer can be given to the questions regarding the influence of G cell population or function on the clinical outcome of ulcer disease and especially the response to medical or surgical therapy

Earlier work in this field (3 4 6 9 12 14 18 20) has largely been concentrated on numerical density of G-cells per area in larger tissue blocks This technique seems inadequate for repeated studies in sizable patient materials which necessitate measurement using endoscopic biopsies The reliability of small biopsies has however been questioned (21) while others find them satisfactory (13)

In this work a method for determination of volume density numerical density and mean volume of G-cells will be evaluated in respect of within and between-observer variation as well as within patient variation The minimum sample size

indicated to be the case for the Stockholm region than for other parts of Sweden is not known There is also a possibility that a higher frequency of thyroid surgery and a higher autopsy rate in these regions might contribute to their higher incidence of malignant thyroid tumors

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he smallest and the three with the largest counts ... between the figures from

population and total G cell volume First the junction of the mucosa of the antrum and that of the body of the stomach was determined by means of multiple sections examined by freeze microscopy After the site of this junction had been ascertained the antral mucosa was stripped from the underlying tissue and the volume determined by submersion into water and measurement of the amount of water thus displaced As this volume includes mucosa and submucosa the fraction of the two components was determined by point counting in the same manner as described for V_V of the G cells

Section thickness was controlled by a Tesatronic Elektronetester (TESA S A Renens Switzerland)

Statistical methods The chi square test was used for all comparisons The figures were placed in a 2 X K table (19) when comparing the values of P_G and the corresponding values of $P_M - P_G$ the values of N and corresponding values of P_M The test for expected values ... of V

RESULTS

Small diffuse areas of intestinal metaplasia devoid of G-cells were observed in slides from both antrum preparations but apart from these areas the distribution of the G-cells was found to be uniform corresponding to the middle part of the mucosa (11) No G-cells were seen in control sections Tissue block *No 4 from antrum No 2 was* unsuccessful as the mucosa was lost during preparation

Figures 1 2 3 and 4 are based on data from antrum No 1 and it can be seen that the minimum sample size determined as the minimum number of P_G giving the accepted levels of the relative standard error is about one hundred *corresponding to a G*

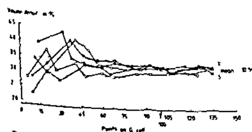


Fig 1 Relationship between *volume density and points* falling on G-cells (↑ 100 points – dispersion between curves are < 10% of the mean value of volume density)

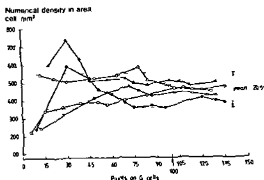


Fig 2 Relationship between numerical density in area and points falling on G-cells (↑ 100 points – dispersion between the curves are <20% of the mean value of numerical density in area)

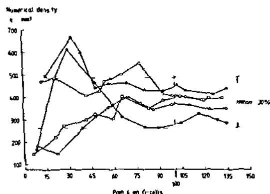


Fig 3 Relationship between numerical density and points falling on G-cells (↑ 100 points – dispersion between the curves is <30% of the mean value of numerical density)

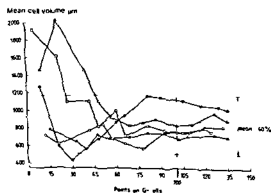


Fig 4 Relationship between mean cell volume and *points falling on G-cells* (↑ 100 points – dispersion between the curves <40% of the mean value of mean cell volume)

necessary for reproducible results will be estimated, and the comparability of results obtained from biopsy material to results obtained from larger tissue blocks will be evaluated Furthermore estimation of the population of G-cells and their total volume from resected antrum preparations will be attempted

MATERIAL AND METHODS

The material comprises 2 preparations from 2 antra No 1 was removed due to recurring duodenal ulcer, and used as it was expected to show a relatively high number and even distribution of the G cells (18) No 2 was removed owing to a gastric ulcer, and chosen because it was reasonable to expect a non uniform distribution of the G cells (22)

Preparation and immunohistochemistry Five tissue blocks of approximately 10 mm in length and 5 mm in width were taken from No 1 Eight tissue blocks of the same size were removed from No 2 and in addition 5 biopsies taken approximately 2 cm from the pylorus using a gastroscopic biopsy forceps

The tissue removed was fixed in 4% methanol free paraformaldehyde and embedded in paraffin The G cells were identified on 5 μm thick sections by means of the indirect immunoperoxidase technique (11) using anti gastrin (2717) raised in rabbits against synthetic human gastrin I (17) in radioimmunoassay this anti serum will determine all four main gastrin components (gastrin 14 17 34 and component I) (16) The method was modified so that the diaminobenzidine was replaced by 3 amino 9 ethyl carbazol (Sigma Chemical Comp St Louis U S A) during the development The same procedure was employed on control sections with the exception of a) the exclusion of anti gastrin serum and b) letting the anti gastrin serum react with gastrin (synthetic human gastrin I Calbiochem Behring Corp U S A) 10 μg per ml diluted anti serum before application to the sections

Morphometry Using a square grid in the ocular of the microscope having 400 points with a mutual distance between them of 0 5 mm (d) and a magnification of 400 a systematic count of points falling on the G cells (P_G) the number falling on the mucosa (P_M) as well as the total number of G cells (N) was carried out on the entire histological slide from each preparation The counting was started at the upper left point falling on the preparation the point being made of horizontal and vertical lines with a distance between them of 1 mm The following variables were then determined

Volume density (V_V) in % $\frac{P_G}{P_M} \times 100$ (23)

Numerical density per unit area (N_A) stated as cells/mm² =

$$\frac{N \times \text{magnification}^2}{P_M \times d^2}$$

Numerical density (N_V) stated as cells/mm³ =

$$\frac{K \times N_A^{3/2}}{\beta \times V_V^{1/2}} \quad (24)$$

The factor K depends on the relative size distribution of the G-cells We used the value 1 07 as the standard deviation in preliminary studies was found to be below 25% of the mean diameter of the G-cells (23). The coefficient β depends on the shape We found the cells ellipsoid with the largest diameter 22 3 ± 2 2 (SD) μm and the smallest 14 3 ± 1 1 (SD) μm Using the diagram of *Weibel* (23) and the values for G-cell diameters we found a value for β of about 1 5

Mean particle volume ($\bar{V}$) in μm³ = $\frac{V_V}{N_V}$

The minimum sample size sufficient for giving an acceptably small variation of the results for the different variables was determined by the cumulative mean plots (10) For this we used the tissue blocks from No 1 We determined the cumulative means of V_V, N_A, N_V and $\bar{V}$ by analyzing an increasing number of microscopic fields from each histological slide Acceptable levels of variation expressed as the relative standard error were arbitrarily fixed at ± 10% for V_V, ± 20% for N_A, ± 30% for N_V and ± 40% for $\bar{V}$ To ensure that the minimum sample size would give acceptable levels for the coefficient of error (CE) this coefficient was determined for the variables with the greatest variation, $\bar{V}$ It can be seen from the formula that $\bar{V}$ only depends on the proportion between P_G and N CE for minimum sample size was calculated from preparation No 1 The formula $CE^2(\Sigma P_G/\Sigma N) = CE^2(P_G) + CE^2(N) - 2r \times CE(P_G) \times CE(N)$ was used where r = the coefficient of correlation between P_G and the corresponding values of N CE for P_G was calculated from the formula

$$\frac{\text{standard deviation of } P_G}{\text{mean value of } P_G \times \text{number of fields}^{1/2}}$$

CE for N was calculated likewise A value of CE below 10% was accepted as satisfactory

Figures for *within observer variation* were obtained by letting the same observer (A) count P_G and P_M twice in the preparation from No 1 The values of P_G and the corresponding values of $P_M - P_G$ from the two counts were compared for calculating the variation In order to ... prepa... united ...) The *homogeneity* of the preparations was evaluated by comparing the different tissue blocks in respect of the values of P_G and the corresponding values of $P_M - P_G$ as well as the values of N and the corresponding values of P_M The figures for V_V, N_A, N_V and $\bar{V}$ from each tissue block from both antra were also compared The samples from No 2 were used to find the minimum number of biopsies to be counted in order to obtain a sample size for P_G sufficient for an acceptably small variation in the estimated variables To test whether this minimum sample size was actually valid for antrum No 2 the values of P_G and the corresponding values of $P_M - P_G$ were compared for the biopsy with the smallest and the one with the largest count for the two with the smallest and the two with the largest counts and the three with

TABLE 3 *Morphometric Data from No 2*

ner	Tissue block No	P_G	P_M	P_M–P_G	N	V_V in %	N_A cells/mm²	N_V cells/mm³ × 10	$\bar{v}$ µm³
	1	106	6390	6284	216	1 66	216	175	949
	2	116	7086	6970	241	1 64	218	178	919
	3	117	6452	6335	223	1 73	214	169	1024
	4	unsuccessful							
	5	63	5013	4950	126				
	6	128	7798	7671	276	1 64	226	188	871
	7	98	5364	5266	209	1 83	249	206	827
	8	105	5303	5198	215	1 98	259	210	941
	Mean ± SD					1 75 ± 0 14 N S	230 ± 19 N S	188 ± 17 N S	922 ± 68 N S
	1	86	5920	5834	189	1 45	204	172	844
	2	106	6787	6681	215	1 56	202	163	956
	3	98	6013	5915	206	1 63	219	180	904
	4	unsuccessful							
	5	60	6764	6704	116				
	6	115	6707	6592	209	1 71	200	154	1113
	7	87	4628	4541	189	1 89	241	193	978
	8	153	8156	8003	308	1 88	242	195	964
	Mean ± SD					1 69 ± 0 18 N S	218 ± 19 N S	176 + 16 N S	960 + 90 N S

ing a reasonable homogeneity When the data for V_V N_A N_V and $\bar{v}$ were compared a statistically significant difference was seen between the values of $\bar{v}$ for the tissue blocks of No 1 (which can be explained by the considerable dispersion tolerated in the estimation of the value of $\bar{v}$) while no differences were seen in the other variables

It can be seen from Table 4 that a minimum of three biopsies are required in order to give values of P_G of approximately one hundred By comparing the values of P_G and the corresponding values of P_M – P_G from the biopsy with the lowest (C) and that with the highest (E) count, as well as the two biopsies with the lowest (A + C) and the two with the highest (B + E) counts statistically significant differences were found When however the biopsies with the lowest (A + C + D) and the three with the highest (B + D + E) counts were compared no difference was seen Neither was any difference found between the figures from the tissue blocks and the figures from the three biopsies with the lowest or the three with the highest counts (Tables 2 and 4) Thus three biopsies were the minimum number giving a reproducible count.

The volume of the stripped antrum mucosa was found to be 11 5 ml The fraction of the mucosa was 91 6 % and of the submucosa 8 4 % of the total volume This gave a mucosal volume of 10 5 ml which using the average value of N_V from the eight tissue blocks (188×10^2 cells/mm³ Table 3 observer A) gave a G-cell population of 197×10^6 When the values of N_V from the biopsies A + C + D and B + D + E (Table 4) were used the population was estimated to be 173×10^6 and 200×10^6 respectively the mean value being 190×10^6 cells When the corresponding figures for

TABLE 4 *Morphometric Data for the Biopsies from Preparation No 2*

Biopsy No	P_G	P_M	P_M–P_G	N	V_V in %	N_A cells/mm²	N_V cells/mm³ × 10²	$\bar{v}$ µm³
A	37	2172	2135	76	1 70	224	186	931
B	43	2200	2157	101	1 95	294	256	762
C	25	2143	2118	40	1 17	119	85	1365
D	38	2034	1996	86	1 86	271	232	801
E	43	3003	2960	74	1 43	158	118	1213
A + C + D	100	6349	6249	202	1 58	204	165	958
B + D + E	124	7237	7113	261	1 71	231	191	895

TABLE 1 *The Coefficient of Error of $\Sigma P_G/\Sigma N$ (Preparation No 1 First Count of A)*

Tissue block No	ΣP_G	ΣN	$\Sigma P_G/\Sigma N$	r	n	CE
1	104	242	0 43	0 898	10	6 4
2	108	261	0 41	0 839	10	7 3
3	109	264	0 41	0 893	10	7 2
4	100	217	0 46	0 651	9	9 9
5	91	171	0 53	0 819	8	7 8

ΣP_G = the summation of P_G values
ΣN = the summation of N values
r = coefficient of correlation between the value of P_G and corresponding value of N
n = number of fields counted
CE = coefficient of error in per cent

cell count of approximately two-hundred (Table 1) The value of CE for $\Sigma P_G/\Sigma N$ was in all five tissue blocks below 10% with this number of P_G

The data from antrum No 1 are seen in Table 2, and from No 2 in Tables 3 and 4

No difference was found, indicating a negligible within-observer variation (coefficient of variation 2%) by comparing the values of P_G and the corresponding values of $P_M - P_G$ for the two counts of observer (A) (Table 2)

In the same manner no differences were found when the values of P_G and the corresponding values of $P_m - P_G$ were compared for observers A and B (Table 3), or the values for A and C (Table 2) indicating a negligible between-observer variation (coefficient of variation 5%)

No difference was found in No 1 when comparing the values of P_G and *the corresponding* values of $P_M - P_G$, as well as the values of N and the corresponding values of P_M from the tissue blocks of both antra, this indicated a high degree of homogeneity In No 2, a statistically significant difference was seen when the figures from tissue block No 5 were included (in this block the P_G count was less than optimal, 60 and 63) With block No 5 excluded, no difference was found, confirm

TABLE 2 *Morphometric Data from No 1*

nter	Tissue block No	P_G	P_M	$P_M - P_G$	N	V_V in %	N_A cells/mm²	N_V cells/mm³ × 10	$\bar{v}$ μm³
	1	127	4147	4020	294	3 06	454	393	779
	2	133	4266	4133	309	3 15	463	399	789
	3	135	4399	4264	335	3 11	494	442	704
t count)	4	136	4255	4119	284	3 20	428	351	897
	5	135	4367	4232	261	3 09	382	302	1023
	Mean ± SD					3 12 ± 0 05 N S	444 ± 42 N S	377 ± 53 N S	838 ± 124 P<0
	1	126	3987	3861		3 16			
	2	135	4141	4006		3 26			
	3	129	4031	3902		3 20			
ond count)	4	119	3684	3565		3 23			
	5	138	4409	4271		3 13			
	Mean ± SD					3 20 ± 0 05 N S			
	1	108	3334	3226		3 24			
	2	143	4206	4063		3 40			
	3	154	4464	4310		3 45			
	4	106	3072	2966		3 45			
	5	128	3951	3823		3 24			
	Mean ± SD					3 36 ± 0 11 N S			

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mean cell volume 922 µm³ (Table 3 observer A) 958 and 895 µm³ (Table 4) were used the total G cell volume was found to be 182 mm³ 166 mm³ and 179 mm³ respectively the mean value being 176 mm³

DISCUSSION

General use of quantitative methods for an investigation of the antral gastrin producing cells depends on the availability of a satisfactory technique

An acceptable consistant examination result is essential for a suitable method and the method should not be too time-consuming

In the present investigation we found using direct microscopy and point counting that the reproducibility was good in respect of within and between observer variation This reproducibility was obtained with a minimum number of G-cells of about two hundred When the figures for this minimum sample size were used we also found good agreement between the counted numbers from single tissue blocks from the same preparation The same was true for figures obtained from biopsy material and tissue blocks

Some dispersion between the figures for mean cell volume was seen We had to accept this from a practical point of view because a smaller relative standard error for the values of the mean cell volume would necessitate an unpracticably high value of the gastrin-cell count The coefficient of error for mean cell volume was below 10% which means that only differences between values for the mean cell volume of more than 10% can be considered as statistically significant in comparative investigations In order to employ the formula of *Weibel & Gomez* (24) for the determination of the numerical density it was necessary to assume that the G cells were of uniform size and shape Further no correction was made for the over estimation caused by the Holmes effect nor for the under estimation due to the inability to recognize and count very small particles (image loss) as we assumed as others that these two effects may cancel out (5 25)

Stave et al (21 22) in contrast to our results found in an investigation based on a resection material that the G cells had a very non uniform distribution within the same antrum Their method gave the number of G-cell profiles per mucosa unit defined as specimens of 6 µm in thickness and 1 mm in width and 6 µm in thickness and 720 µm in width The great variation within the units can be explained by the fact that these individual units may contain too few G-cells for a reliable and reproducible estimate (Fig 2)

Our results in respect of the use of biopsy material for quantitation of G-cells are in agreement with *Hobbs & Polak* (8) as well as *Polak et al* (13) However their method cannot be employed in daily routine work as the results were obtained by means of analysis in a specially constructed »television Image Analyzer« which gives the results as the number of cells per measuring unit They cannot measure the volume density numerical density nor the mean cell volume by this method

Biopsy material permits the data of N_A and N_V to be expressed as density Determination of the total G-cell population or the total G-cell volume will however require a resection material comprising the whole antrum The method described by *Keuppens et al* (9) and *Roystone et al* (18) in which the area of the antrum is determined by planimetry necessitates complete and uniform stretching of the preparation which we found difficult to achieve The formula of *Abercrombie* (1) for the determination of the number of G-cells has the limitation that it is truely applicable for spherical particles only (23) Our method with the determination of the volume is simple but gives over-estimated values as we do not correct for tissue shrinkage

CONCLUSIONS

This investigation demonstrates that density studies of the human antral gastrin producing cells are possible and that biopsy material can be used when simple precautions are taken in respect of the sample size A simple method is described for the estimation of the G-cell population and the total G cell volume in resected antra

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The anti gastrin serum was kindly donated by Professor J F Rehfeld Aarhus The authors wish to thank Professor *D Andersen* Odense for permission to use the antrum preparations and for help in preparation of the manuscript The work was supported by the *Danish Medical Research Council* (Journal number 552 944)

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Acta path microbiol scand. Sect A 88 263–268 1980

EFFECTS OF A CHEMOTACTIC FACTOR AND *BACTEROIDES FRAGILIS* LIPOPOLYSACCHARIDE ON BONE RESORPTION IN TISSUE CULTURE

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Sveen, K. Effects of a chemotactic factor and *Bacteroides fragilis* lipopolysaccharide on bone resorption in tissue culture Acta path microbiol scand Sect A 88 263–268 1980

Release of previously incorporated ^{45}Ca from fetal rat bone in tissue culture was stimulated by preparations of the polymorphonuclear leukocyte chemotactic factor isolated from lipopolysaccharide (LPS)-induced inflammatory exudate in rabbits as well as by *Bacteroides fragilis* LPS High concentrations of released hydroxyproline and lactate seemed to correlate well with a high percentage of ^{45}Ca liberated into the culture medium An active bone resorption was stimulated by a concentration of 1 µg/ml of the chemotactic factor The peak in amount of released ^{45}Ca was at a concentration of 5 µg/ml of the chemotactic factor (LPS-CF) as well as of the LPS preparation whereas the parathyroid hormone was most active at 1 IU/ml Their effect was connected with the formation of osteoclasts Neither LPS-CF nor LPS stimulated a release of ^{45}Ca or hydroxyproline from heat-devitalized bones Heparin added to LPS-CF did not enhance its resorptive potential whereas when added to LPS it had a synergistic effect It is suggested that the bone resorptive effect exerted by LPS may be caused by chemotactic factors elaborated by activation of the complement system and that these factors may be of importance in the pathophysiology of periodontal disease

Key words Bone resorption chemotactic factor *Bacteroides fragilis* lipopolysaccharide

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Received 25 i 80 Accepted 15 iii 80

Gradually increasing mobility of teeth is caused by the loss of bone supporting tooth roots Epidemiological studies clearly demonstrate a positive correlation between the severity of periodontal disease and the amount of bacterial and microbial products in the gingival region (1) However the pathways connecting these microorganisms with the characteristic tissue-destructive mechanisms of periodontal disease are not well understood Microbial products and components originating from degraded cells enter the soft and hard tissues These either directly activate mechanisms destructive to the tissue or indirectly trigger defence mechanisms which in turn might generate mediators destructive to the same tissue

Bacterial lipopolysaccharides (LPS) have been found to stimulate bone resorption in *in vitro* tissue cultures (7 8) Also the supernatant after centrifugation of immune complexes has been reported to induce bone resorption and this was suggested to be due to some activated complement components (3)

This report is concerned with studies utilizing a previously characterized complement-derived chemotactic factor (16 17) The aim of the study was to find out whether the chemotactic factor induced bone resorption and if so to what extent this activity was comparable to that of LPS isolated from *Bacteroides fragilis* ss *fragilis* strain Lille 323 and of parathyroid hormone

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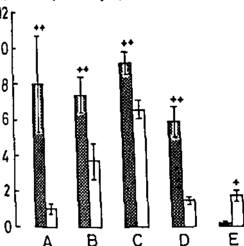


Fig 2 Effect of optimal doses of LPS CF LPS and of heparin added to LPS on the release of lactate from fetal rat bones cultured for 48 h in Bigger s modified medium Each column represents the mean amount (mmol) of lactate per litre culture medium ± standard deviation (vertical bar) from six test bones Closed columns illustrate culture medium + test substance open columns culture medium alone Test cultures contain per ml of medium A) 5 µg LPS CF B) 5 µg LPS C) 5 µg LPS + 10 IU heparin and the control medium 5 µg of LPS D) 1 IU PTH E) 10 IU heparin +P > 0 05 = not statistically significant + +P ≤ 0 025

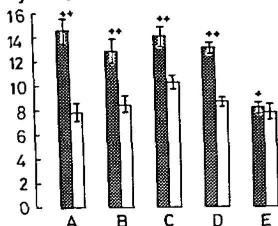


Fig 3 Effect of optimal doses of LPS CF LPS PTH and of heparin alone and added to LPS on the release of hydroxyproline from fetal rat bones cultured for 48 h in Bigger s modified medium Each column represents the mean amount (µg) of hydroxyproline per ml of culture medium ± standard deviation (vertical bar) from six test bones Closed columns illustrate culture medium + test substance open columns medium alone Test cultures

giving optimal response at a specific dose (Fig 1)

most active at a concentration of 1 IU per ml of the medium The activity of LPS-CF was considerably higher than that of LPS on a weight basis and showed values of ^{45}Ca liberated significantly above that of the controls at all dilution steps tested

Heparin alone induced no release of ^{45}Ca but when heparin was added to LPS there was a pronounced increase in the release (p = 0 025) (Ta

TABLE 1 *Effect of Adding Heparin to Bigger s Modified Medium Containing LPS on the Release of ^{45}Ca Lactate and Hydroxyproline from Living Fetal Rat Bone and the Effect of LPS on Devitalized Fetal Rat Bone in Tissue Culture*

Treatment of bone	^{45}Ca (%) (mean ± s d)	P[a]	Hydroxyproline (µg/ml) (mean ± s d)	P	Lactate (mmol/l) (mean ± s d)	P
Heparin 10 IU/ml	4 46 ± 2 96[b]		8 33 ± 0 18		0 14 ± 0 19	
LPS 5 µg/ml	9 56 ± 1 74	0 025	10 17 ± 1 65	0 025	6 51 ± 1 07	0 025
Heparin 10 IU/ml + LPS 5 µg/ml	13 43 ± 3 86	0 025	13 82 ± 2 03	0 025	9 15 ± 1 49	N S
70 °C for 8 min	3 20 ± 0 87		4 99 ± 1 19		N D[d]	
70 °C for 8 min + LPS 5 µg/ml	4 17 ± 0 85	N S[c]	4 97 ± 1 30	N S	N D	

[a] Values of P ≤ 0 05 were accepted as statistically significant, mean calculated from 4–6 samples
[b] Difference statistically significant to control
[c] N S = not significant
[d] N D = not detected

MATERIALS AND METHODS

Test Materials

LPS (endotoxin) was isolated from *Bacteroides fragilis* ss *fragilis* strain Lille E 323 by phenol-water extraction (20) and purified by ultracentrifugation and treatment with ribonuclease and deoxyribonuclease as described before (12) The complement-derived chemotactic factor was elaborated *in vivo* by the injection of LPS-E 323 suspensions into wound chambers implanted subcutaneously in rabbits as described earlier (18), and the inflammatory exudate sampled 3 h later The fractionation procedure of the LPS-induced inflammatory exudate and the isolation of the chemotactic factor (LPS-CF) has been reported in detail (16) Heparin was purchased from A/S Apothekernes Laboratorium for Specialpræparater, Oslo, and parathyroid hormone used as a positive control, was supplied by Lilly, Indianapolis, Ind, USA

Animals

Female rats from an inbred Wistar strain were used On the eighteenth or nineteenth day of pregnancy they were injected subcutaneously with 100 μCi of ^{45}Ca One day later the rats were killed, the abdomen washed with 0 05 per cent Pyricept® followed by 5 per cent iodine in ethanol The fetal ulnae and radii were removed and explanted as pairs for organ culture

Culture Medium

Bigger s modified medium (Flow Laboratories, Irvine Scotland) containing 0 1 per cent of bovine serum albumin (Armour Pharmaceutical Company Ltd, England) was used throughout No antibiotics were employed

Culture Method

Two bones from the fetus were used as controls and the two others as experimental bone The explanted bones were precultured for 24 h in 0 5 ml of the culture medium Thereafter experimental radii and ulnae were cultured for 48 h in the same volume of the culture medium containing test materials In one experiment one ulna and one radix from the same fetus were used as experimental bone and the contralateral ulna and radix were used as controls (Table 2) Cultures were maintained at 37 °C in an atmosphere of 5 per cent CO_2 20 per cent O_2 and 75 per cent N_2

Quantification of Released ^{45}Ca

After cultivation of the bones for 48 h, 0 1 ml of the culture medium was prepared for liquid scintillation counting of ^{45}Ca The bones were then demineralized in 0 5 ml of 1N HCl for 24 h, and 0 1 ml of the HCl extract was prepared for liquid scintillation The amount of ^{45}Ca released from the bones during culture was expressed as the percentage of the total radioactivity initially present, calculated from the total counts per minute (cpm) in the bone In one experiment, the amount of ^{45}Ca released was expressed as the ratio between the ^{45}Ca released (cpm) from the treated bones and from the comparable control bones (Table 2)

Quantification of Lactate and Hydroxyproline

The lactate concentration of the test culture and t control medium was determined by the reduction nicotinamide adenine dinucleotide with lactic dehydro nase (Boehringer, GmBh Mannheim, West-German and measured spectrophotometrically Free hydroxypr line (OH-proline) was estimated in both media accordi to the method of *Dabew & Struck* (2)

Histological Examination

Bones were fixed in 10 per cent formalin in phosph buffered saline, pH 7 2 The bones were then demineral zed in 0 1 M EDTA overnight and embedded paraffin Longitudinally cut sections of 5 μm thickn were stained with hematoxylin and eosin

Statistical Methods

For evaluation of the difference between the te ... as to the amount ... was performed according to the *t* test (4)

RESULTS

The release of ^{45}Ca from prelabelled embryonic r bones into the culture medium was effected by th materials tested over a certain concentration rang

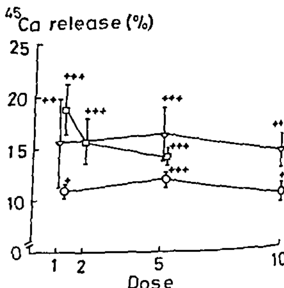


Fig 1 Effect of different concentrations (μg/ml Bigger s modified medium) of chemotactic factor (LPS CF) and lipopolysaccharide (LPS) and of parathyroid hormone (PTH) (IU/ml) on the release of previously incorporated ^{45}Ca from fetal rat bones cultured for 48 h Each point represents the mean percentage ± one standard error of the mean (vertical bar) from six test bones For calculation of bone resorption, see Materials and methods –▽– LPS CF, –O– LPS –□– PTH Values of $P > 0\,05$ = statistically not significant to control $++P \leq 0\,05$, $+++P \leq 0\,025$

percentage of ^{45}Ca mobilized from the same fetal bone by the stimulant used Also high concentrations of lactate in the culture medium seemed to correlate well with a high percentage of ^{45}Ca liberated as well as with the amount of OH proline released Each of the parameters used thus could reflect the magnitude of the osteoclastic activity induced by the special stimulant used

Morphologically LPS-CF and LPS caused a proliferation of osteoclasts and a removal of the matrix as shown by others using LPS isolated from *Bactero des melaninogen cus* (7) and from *Escheri ch a coli* (8) These stimulated osteoclasts also contain a greater number of nuclei than those seen in the controls using LPS (5) and PTH (11) as stimulants

Large doses of LPS-CF in the culture medium resulted in a decreased release of ^{45}Ca This was also demonstrated with high doses of LPS and PTH which would suggest the occurrence of either an inhib tory mechanism or a toxic effect Neither LPS-CF nor LPS stimulated ^{45}Ca release beyond that of PTH when tested on the peak of their bell shaped dose response curves It is likely that the inductory mechanism of LPS CF also is quite d ffere

may also bind to the membrane by the opening of the lipid bilayer which would allow interaction of the fatty acids Results (unpublished) indicate however that the main part of the bone resorptive activity after the splitting of the *Fusobacterium* LPS with 1 per cent acetic acid was retained in the supernatant and not in the pellet. This may indicate that lipid A is not the only portion of the LPS molecule in which the potential for biological activities are integrated This is in keeping with other studies (12 13 14 16 19)

The addition of heparin to the culture medium significantly increased the resorptive activity of LPS as has also been shown by others (8) whereas no such effect was found when heparin was added to the culture medium containing LPS-CF The mechanism of this synergistic effect is unknown although heparin is known to activate the classical complement pathway and thus induce the elaboration of complement-derived factors which may be active in stimulation of bone resorption Why heparin did not enhance the bone resorptive effect induced by LPS-CF is also unknown

LPS have a lipid A qu te different from that of *Fusobacterium* LPS It has been put forward that this part of the endotoxin molecule is responsible for the biological activities exerted by LPS (6) Also lipid A has been suggested to be responsible for the active ^{45}Ca release from bone (9) It is known that lipid A activates the classical complement pathway and that the polysaccharide portion initiates the activation of the alternative pathway *B fragilis* LPS has been shown to activate the alternative pathway (15) resulting in the elaboration of the complement derived chemotactic factor C5a (16)

There is no proof that embryonic bone does or does not contain endogenous complement factors It may be suggested therefore that complement factors are available inside as well as outside the bone and are activated by LPS resulting in the elaboration of chemotactic factors It is not likely that LPS-CF and LPS which are each unique molecular entities should react with the same receptor in the cell membrane of the osteoclast and initiate the biochemical events resulting in bone resorption It is more likely that these events are mediated by the elaboration of the chemotactic factor C5a binding to receptors in the cell membrane However LPS molecules via the lipid part

number of osteoclasts whereas the effect of LPS causes changes in cell calcium as well as an increase in the number of nuclei of the osteoclasts The dynamics behind the inductory mechanism of calcium release by LPS as well as by LPS-CF are uncertain However neither calcium nor cAMP needs to be the final mediator of bone resorption

The potentiating effect of heparin on LPS from *B fragilis* is interesting in that no correlation between the number of mast cells and heparin content of inflamed gingival tissues has been found (21) which indicates that mast cells have degranulated This also is supported by the finding that severe inflamed gingiva contain fewer mast cells than healthy gingiva do Furthermore a stimulation of the synthesis of mast cells in the connective tissue adjacent to the gingival microbial plaque has been indicated (22) It may thus be concluded from the results presented that the synergistic effect of heparin on endotoxin as well as the activity of the LPS induced complement-derived chemotactic factor on bone resorption could be significant factors in the pathophysiology of periodontal disease

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TABLE 2 *Effect of Adding Heparin to Bigger s Modified Medium Containing Chemotactic Factor (LPS CF) on Release of ⁴⁵Ca and Hydroxyproline from Fetal Rat Bone in Tissue Culture*

Treatment of bone	Ratio treated to control[a]			
	^{45}Ca (mean ± s e)	P[b]	Hydroxyproline (mean ± s e)	P
LPS *CF* 5 μg/ml	1 13 ± 0 05	<0 05	1 52 ± 0 16	<0 05
LPC CF 5 μg/ml + 10 IU/ml heparin	1 14 ± 0 08	<0 05	1 52 ± 0 15	<0 05

[a] Release ratios of ^{45}Ca and hydroxyproline are calculated from four pairs of cultures in each experiment
[b] P values based on a difference of a ^{45}Ca and hydroxyproline release ratio from 1 0

ble 1) The difference in the release of ^{45}Ca from prelabelled experimental bones and control bones was not statistically significant

Except for heparin (Table 1) all the test materials examined in doses giving optimal ^{45}Ca release into the culture medium also induced the liberation of lactate, which was significantly higher than in the medium of their respective controls (Fig 2)



giant cells (arrows) ... Both sections are oriented with the periosteum at the upper edge Hematoxylin and eosin ×488

However, the concentration of lactate in medium containing heparin only was insignificant although it seemed to potentiate the release of lactate into the medium containing LPS (p = 0 025) (Table 1)

A significant release of OH proline into the culture medium containing LPS CF LPS and PTH in optimal concentrations was demonstrated (Fig 3) Ten units of heparin did not affect the release of OH proline (Table 1) but the same concentration of heparin enhanced the effect of LPS significantly above that induced by LPS alone (p = 0 025)

The activity of LPS CF to induce the liberation of ^{45}Ca and OH proline into the culture medium with or without heparin added, gave values significantly above that of the controls (p <0 05) (Table 2) However, heparin did not potentiate the capacity of LPS CF to induce either the release of ^{45}Ca or OH proline into the culture medium (p >0 05)

Fetal bones cultured for 48 h with LPS-CF or LPS showed an increase in cell density with the finding of multinuclear cells close to the bone as well as a scarcity of bone matrix as compared to control bones (Fig 4)

DISCUSSION

Preculture of fetal rat bone under strictly defined conditions in culture medium only for 24 h contributes to an exchange of loosely complexed ^{45}Ca with the stable calcium Both metabolic and passive exchange mechanisms are primarily responsible for the release of ^{45}Ca from prelabelled rat bone in *in vitro* cultures (9) The magnitude of the passive exchange component may be estimated from prelabelled heat-devitalized bone in the culture medium Neither LPS-CF nor endotoxin had any effect on the exchange mechanism of devitalized bone

A large amount of OH proline liberated from the bone matrix was found to be related to a high

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THE INTRA-LESIONAL VARIATION OF TYPE, LEVEL OF INVASION, AND TUMOUR THICKNESS OF PRIMARY CUTANEOUS MALIGNANT MELANOMA

55 Malignant Melanomas Studied by Serial Block Technique

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Søndergaard K The Intra lesional variation of type level of invasion and tumour thickness of primary malignant melanoma 55 malignant melanomas studied by serial block technique Acta path microbiol scand Sect A 88 269–274 1980

In order to evaluate the prognostic importance of type of melanoma, level of invasion and tumour thickness in a retrospective study of cutaneous melanomas it is prerequisite that the available histological material be representative To investigate this condition 55 primary cutaneous melanomas were studied randomly by serial block technique The histological classification of each tissue block as to 1) type of melanoma 2) level of invasion and 3) tumour thickness was correlated with the overall classification of the corresponding whc[illegible] melanoma lentigo maligna melanoma [illegible] and arranged in 6 [illegible] nm and more than 6 00 mm) The study revealed a consistency of 95% (52/55) between the type of the central cross section and the type of the whole tumour In 96% (53/55) of the central cross sections the level of invasion was equal to that of the whole tumour In 95% (52/55) of the central cross sections the tumour thickness was situated in the same group as that of the whole tumour It is concluded that type level and thickness of malignant melanomas can be evaluated in retrospective studies if central cross sections of the melanomas are available for histological examination

Key words Malignant melanoma cutaneous classification

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Accepted as submitted 3 iii 80

The most widely used histological parameters in the prognostic evaluation of primary cutaneous malignant malanoma are the types of melanoma and the le[illegible]

evaluate the frequency of incorrect histological grouping when only selected parts of the lesions are examined microscopically as may be the case in retrospective studies

MATERIAL AND METHODS

The material consisted of 55 cutaneous melanomas operated at the Finsen Institute during the period July 1976 to June 1977 The size of the le[illegible]
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Fig 2 A section from the peripheral part of a polypoidal malignant melanoma graded as NM level II The polypoidal part of the tumour is massively infiltrated by melanoma cells and the upper surface is ulcerated (arrows) Haematoxylin and eosin ×14

tumour cells were present there the block was typed NM

2) *Level of invasion* was graded according to the description by *Clark et al* (5) as shown in Fig 3 Level I indicated that the tumour cells were confined to the epidermis Level II indicated tumour cells which extended into the papillary dermis but did not reach the reticular dermis The level III tumour cells impinged upon the interface between the papillary and the reticular dermis but did not invade the reticular dermis Level IV designated tumour cells clearly extending between the collagen bundles of the reticular dermis and the level V tumour invasion progressed into the subcutaneous fat

3) *Tumour thickness* was measured on the histologic sections by means of an ocular micrometer as the maximal distance in mm from the top of the epidermal

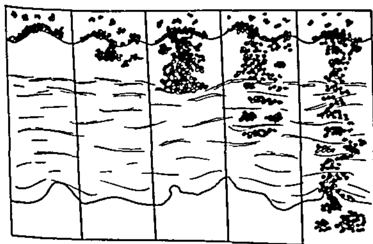


Fig 3 The levels of invasion used in this study

the macroscopic photos of each lesion The blocks were embedded in paraffin and 4 to 6 haematoxylin and eosin stained sections were prepared from each block

The histological sections were mixed and randomly studied by light microscopy Without the help of any clinical or other informations each block was classified as to 1) type of melanoma 2) level of invasion and 3) tumour thickness

Criteria and Definitions

1) *Type of melanoma* The following groups were used in the typing of the blocks

Superficial spreading melanoma (SSM) characterized by an intra epidermal Pagetoid extension of atypical melanocytes When dermal invasion was present the intra epidermal spread of tumour cells extended more than 3 rete ridges from areas of clear dermal invasion (5)

Lentigo maligna melanoma (LMM) characterized by a continuous proliferation of pleomorphic melanocytes in the basal layer of the epidermis Epidermal atrophy and solar dermal elastosis were present (5) The group included non invasive lesions (i e lentigo maligna) as well as invasive melanomas

Acral lentiginous melanoma (ALM) characterized by lentiginous intra-epithelial proliferation of pleomorphic tumour cells extending more than 3 rete ridges from areas of dermal invasion Marked acanthosis and elongation of rete ridges was present (9)

Nodular melanoma (NM) being an invasive melanoma with peripheral intra epidermal spread of tumour cells extending less than 4 rete ridges from areas of dermal invasion (5)

Melanomas of unclassifiable type designating melanomas not included in the above mentioned groups

Nevoid lesions designating blocks containing too little tumour tissue for the diagnosis of invasive or non invasive melanoma to be made

The serial blocking of polypoidal melanomas (Fig 1) resulted in a number of blocks without visible connection between the polypoidal part of the melanoma and the skin (Fig 2) The typing of such blocks was based on the growth pattern of tumour in the underlying skin If n

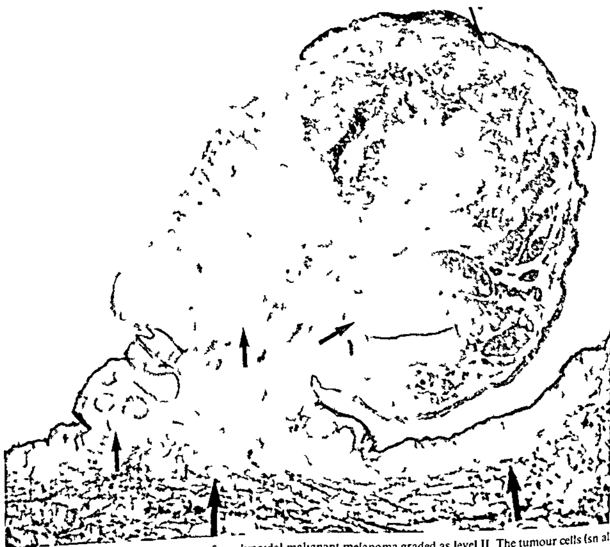


Fig 1 A section from the central part of a polypoidal malignant melanoma graded as level II The tumour cells (small arrows) do not reach the interface between the papillary and the reticular dermis (large arrows) Haematoxylin and eosin × 14

TABLE 3 *The Tumour Th ckness of 55 Melanomas and the Rate of Consistency between Tumour Thickness of the Central Block and that of the Whole Tumour Correlated to the Type of Melanoma*

The overall type of 55 melanomas		The overall thickness of 55 melanomas (mm)						Central blocks with thickness con sistent with that of the whole tumour		
Type	Number	0– 0 75	0 76– 1 50	1 51– 2 25	2 26– 3 00	3 01– 6 00	>6 00	Number	%	95% confidence limits
SSM	44	6	6	9	7	11	5	42	95	85– 99
LMM	4	1	0	0	0	1	2	4	100	40–100
ALM	1	0	0	0	0	1	0	1		
NM	6	0	0	0	1	2	3	5	83	36–100
Total	55	7	6	9	8	15	10	52	95	85– 99

vel of invasion was consistent with that of the hole tumour (Table 2) whereas the level in 2 locks diverged from that of the whole tumour In 5% (170/261) of all blocks the level agreed with iat of the whole tumour In 91 blocks coming redominantly from the peripheral parts of the s ons the level diverged from that of the whole imour

Tumour th ckness Table 3 shows the distribution f the 55 melanomas according to the thickness of ie whole tumour (7 lesions 0–0 75 mm 6 lesions 76–1 50 mm 9 lesions 1 51–2 25 mm 8 lesions 26–3 00 *mm* 15 lesions 3 01–6 00 mm and 10 sions more than 6 00 mm) In 95% (52/55) of ie central blocks the tumour thickness was in the ame group as that *of the whole tumour (Table 3)* n 2 central blocks the thickness diverged from that f the whole tumour In 65% (169/261) of all the locks the *thickness* corresponded with that of the hole tumour In 92 blocks coming predominantly rom the peripheral parts of the melanomas the hickness diverged from that of the whole tumour

DISCUSSION

ype of melanoma In 1969 *Clark et al* (5) lemonstrated the prognostic significance of a istologic classif cation of primary cutaneous mela iomas into 3 types (SSM LMM and NM) Recently Reed (9) found that melanomas on the palm of the and and on the sole of the foot diverged istologically from Clark s 3 types of melanoma ind he proposed the new subtype named ALM SSM LMM and ALM are characterized by l fferent patterns of intra-epithelial proliferation of umour cells in the periphery of the lesions in contrast to NM However the type of melanoma is unclassifiable in a number of cases The more tissue blocks available from each tumour the fewer unclassifiable lesions (6) In the present study 95% (52/55) of the central blocks showed a type cons s en w h h f h l of the central cross section and that of the whole tumour

Level of invasion In 1969 the prognostic importance of the grading of the depth of invasion of cutaneous melanomas on 5 levels (Fig 3) was demonstrated by *Clark et al* (5) However this method involves some problems *Suffin et al* (10) tested the comparability of grading of the depth of invasion done by a number of pathologists using *Clark* s levels An initial disagreement was found regarding the classification of level II *and level III* and agreement was reached only after instruction by Dr *W H Clark* In the present study the level of invasion of 96% (53/55) of the central blocks was consistent with that of the whole tumour *This* corresponds reasonably with the impression of *Hermanek et al* (7) that a few sections *from the* most prominent part of a melanoma are sufficient to classify the level of invasion

Tumour thickness In 1970 *Breslow* (1) showed *that the* prognosis of cutaneous melanomas depen ded on their thickness defined as the greatest distance in mm from the epidermal granular cell layer to the deepest invasion of tumour 5 groups were used (0–0 75 mm 0 76–1 50 mm 1 51–2 25 mm 2 26–3 00 mm and more than 3 00 mm) (1) and *the* greater the thickness the poorer the prognosis (1 2) A problem of this method is that an artificial increase in tumour thickness will be measured if the tumour is not cut at 90 degrees to the surface of the skin A gross error of 22 5

granular cell layer to the deepest point of tumour invasion, as *described by Breslow* (1, 2) The thickness was measured at 90 degrees to the skin surface (4) In *ulcerated lesions* the thickness was measured from the most superficially placed tumour cells in the ulcer base to the deepest tumour invasion (2, 4)

In blocks without visible connection between the polypoidal part of a melanoma and the skin (Fig 2) the thickness was measured from the granular cell layer of the upper epidermal lining of the polypoidal part to the deepest invasive tumour cells in the polypoidal part If a greater distance was found from the epidermal granular cell layer of the underlying skin to tumour cells invading the corium or the subcutis, this distance was used

In melanomas without dermal invasion the thickness was designated as 0 mm In the evaluation of the results 6 groups were used 0–0 75 mm, 0 76–1 50 mm, 1 51–2 25 mm, 2 26–3 00 mm, 3 01–6 00 mm, and more than 6 00 mm

Finally the *overall type of melanoma level of invasion and tumour thickness* were designated The overall type of each of the 55 melanomas was found by reviewing all the sections of each melanoma The overall level of invasion and the overall tumour thickness of the whole tumour were indicated as the deepest level and the greatest thickness found in one or more sections of the tumour

In the present study the classification of the melanomas was based on the principles demonstrated and discussed by Dr *W H Clark* and Dr *A Breslow* (11)

RESULTS

The material consisted of 10 polypoidal melanomas (Fig 1) and 45 non-polypoidal melanomas The serial blocking resulted in 261 tissue blocks In 10 tissue blocks no connection was seen between the polypoidal part of the melanoma and the skin (Fig 2)

Type of melanoma Based on all the blocks the material included 44 SSM, 4 LMM, 1 ALM and 6 NM (Table 1) In 95% (52/55) of the lesions t type of melanoma based on the central block w
[illegible]
[illegible]
[illegible]
blocks the type was in agreement with that of t whole tumour In 26 blocks the type was differe from that of the whole tumour *11 blocks from t* peripheral parts of the melanomas were graded nevoid lesions, 8 blocks showed melanomas unclassifiable type, whereas 7 blocks showe melanomas without lateral intra-epidermal tumo invasion although they originated from melanom classified as SSM and LMM

Level of invasion By using all the blocks th following distribution of the 55 melanomas wa found 1 lesion level I, 4 lesions level II, 21 lesion level III, 21 lesions level IV, and 8 lesions level V (Table 2) In 96% (53/55) of the central blocks th

TABLE 1 [illegible]

The overall type of each melanoma		Central blocks with type consistent with that of the whole tumour		
Type	Number	Number	%	95% confiden limits
SSM	44	42	95	85– 9
LMM	4	3	75	19– 9
ALM	1	1		
NM	6	6	100	54–10
Total	55	52	95	85– 9

TABLE 2 *The Level of Invasion of 55 Melanomas and the Rate of Consistency between the Level of Invasion of the Central Block and that of the Whole Tumour*

The overall type of 55 melanomas		The overall level of 55 melanomas					Central blocks with levels consistent with that of the whole tumour		
Type	Number	I	II	III	IV	V	Number	%	95% confidence limits
SSM	44	0	4	19	17	4	43	98	88–100
LMM	4	1	0	0	1	2	4	100	40–100
ALM	1	0	0	0	1	0	1		
NM	6	0	0	2	2	2	5	83	36–100
Total	55	1	4	21	21	8	53	96	87–100

MALIGNANT MELANOMA OF THE FOOT

A Clinicopathological Study of 125 Primary Cutaneous Malignant Melanomas

KNUD SØNDERGAARD and GRETE OLSEN

Department of Pathology and Department of Plastic Surgery the Finsen Institute Copenhagen Denmark

Søndergaard K. & Olsen G Malignant melanoma of the foot. A clinicopathological study of 125 primary cutaneous malignant melanomas Acta path microbiol scand Sect. A 88 275–283 1980

In a retrospective study of 125 primary cutaneous malignant melanomas of the foot treated from 1949 to 1977 the prognostic importance of various histological and clinical factors was observed Clinical stage location of tumour on the foot, level of invasion tumour thickness mitotic rate and presence of ulceration correlated well with survival while sex and histological type did not A histological transition was found between the superficial spreading type and the acral lentiginous type indicating a close biological relationship between the two types of melanoma

Key words Malignant melanoma cutaneous foot, classification

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Accepted as submitted 3 iii 80

Various studies have found primary malignant melanoma of the foot to account for 3–15% of the total number of primary skin melanomas (11 15 16 19 21) In 1969 *Clark et al* (5) demonstrated the prognostic significance of a histological classification of primary cutaneous melanoma into 3 types (superficial spreading melanoma, nodular melanoma, and lentigo maligna melanoma) Recently *Reed* (24) described a new type of melanoma acral lentiginous melanoma on plantar and palmar surfaces

A clinicopathologic study of 125 patients with malignant melanoma of the foot was undertaken in order both to investigate the frequency and prognostic importance of the new type of melanoma described by *Reed* and to estimate the prognostic significance of the following clinical and histological variables Clinical stage sex site of tumour on the foot, type of melanoma according to *Clark* level of invasion according to *Clark* tumour thickness according to *Breslow* mitotic rate and presence of ulceration.

MATERIAL AND METHODS

From 1949 to 1977 149 patients were operated on at the Finsen Institute for primary cutaneous malignant melanoma of the foot. In 126 patients the tumour was primarily excised whereas 23 patients were admitted for extended excision after primary excision elsewhere Inguinal lymph nodes were dissected where clinically enlarged Postoperatively the patients received frequent follow up examinations at the Finsen Institute (the Radium Centre and the Department of Plastic Surgery) for up to 10 years or more.

In 125 patients the histological material from the primary lesion was suitable for the microscopic classification which was made by one of the authors (K.S.) The histological sections were stained by haematoxylin and eosin

The Clinical Reporting of Patients with Melanoma of the Foot Included

1) *Site of tumour* a) sub-/parungual region b) planta pedis and c) dorsum pedis comprising the rest of the foot.

2) *Sex*

3) *Clinical Stage* as described by *Olsen* (19 20)

degrees increases the apparent thickness by 9% (3) In the present study a 6th group was used (more than 6 00 mm) in order to separate very thick melanomas By using these 6 groups 95% (52/55) of the central blocks showed a tumour thickness consistent with that of the whole tumour

Conclusion The present study demonstrates a 4–5% inconsistence between the central block and the whole tumour with regard to the classification of type level and thickness This means that it is possible to evaluate the prognostic significance of type level and thickness in retrospective studies provided that central cross sections are available for the histological examination It can also be concluded that selected parts of a cutaneous melanoma may be removed for electron microscopic examination or other tissue analyses with only a minimal risk of interference with the histological classification if the most elevated part of the lesion is reserved for the microscopic evaluation

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Fig 1 Malignant melanoma, superficial spreading type (SSM) Planta. Marked Pagetoid extension of tumour cells into the hyperplastic epidermis is seen No dermal invasion is found in this area. Haematoxylin and eosin X 130

Fig 2 Malignant melanoma, acral lentiginous type (ALM) Sub-/parungual region A lentiginous pattern of tumour cells is seen in the hyperplastic epidermis No dermal invasion is found in this area. Haematoxylin and eosin. X 130

Fig 3 Malignant melanoma, histologically intermediate between superficial spreading melanoma and acral lentiginous melanoma (SSM ALM) Planta In this area of tumour moderate Pagetoid extension of tumour cells is present as well as lentiginous growth pattern and epidermal hyperplasia. Haematoxylin and eosin. X 130

Fig 4 Malignant melanoma, nodular type (NM) Dorsum. No adjacent intra-epidermal extension of tumour cells is present Haematoxylin and eosin. x 130

Stage I No clinical signs of metastases

Stage II Clinically enlarged and suspicious inguinal lymph nodes and/or a few cutaneous metastases close to the primary lesion

Stage III Other signs of metastases i e further groups of lymph nodes cutaneous metastases along the lymphatic trunks remote cutaneous metastases and visceral and cerebral metastases

The Histological Evaluation of the Melanomas Included

1) *The histological type of melanoma* according to *Clark et al* (5) and *Reed* (24) as previously described by *Søndergaard* (26)

Superficial spreading melanoma (SSM) (Fig 1)
Lentigo maligna melanoma (LMM)
Acral lentiginous melanoma (ALM) (Fig 2)
Nodular melanoma (NM) (Fig 4)
Melanoma of unclassifiable type

2. Level of invasion was graded according to *Clark et al* (5) as described by *Søndergaard* (26) Level I ~ epidermis, level II ~ papillary dermis level III ~ papillary – reticular interface level IV ~ reticular dermis and level V ~ subcutis Polypoidal melanomas in which tumour cells did not reach the papillary – reticular interface were named level PM II

3 *Tumour thickness* was measured in mm from the top of the epidermal granular cell layer to the deepest point of tumour invasion as described by *Breslow* (3 4)

4 *Mitotic rate* In at least 1 mm² (using high power fields X 400) the mitoses were counted and converted to 1 0 mm² by πr² In our microscope the area of 10 high power fields (diameter 0 35 mm) was 0 96 mm² i e very close to 1 mm²

5 *Ulceration* Size of ulceration if any was measured in mm by an ocular micrometer on the histological cross section of the tumour

Statistical Analysis

The cumulative survival rates were calculated by the life table method and compared by the Logrank test (2 23) Where appropriate Chi² test was used Significance was assessed at P less than 0 05

RESULTS

In 125 patients the histological material from the primary lesion was available and suitable for microscopic classification From each tumour 1–5 cross sectional tissue blocks including the central part of the tumour were preserved.

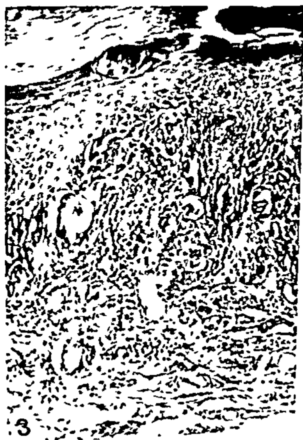
The age of the patients varied from 22 to 94 years (mean value 60 years) 45 patients (36%) were under and 80 (64%) over 55 years of age

Site of melanoma 14 melanomas (11%) were localized sub-/parungually 50 (40%) at planta and 61 (49%) at dorsum The 10 year survival rates were Sub-/parungual 21% planta 40%, and dorsum 55% (Fig 5a) In patients with sub-/parungual melanoma the survival rate was significantly lower than in patients with lesions of the rest of the foot ($X^2 = 4\ 073$ df 1 $P < 0\ 05$) In patients with melanoma at planta the survival rate was lower than in patients with melanoma at dorsum but the difference was not significant ($X^2 = 1\ 552$ df 1 $P > 0\ 2$)

TABLE 1 *Sex of 125 Patients with Melanomas of the Foot Correlated to the Site of Tumour*

Sex	Sub /parung		Planta		Dorsum		Total	
	No	%	No	%	No	%	No	%
Male	5	36	17	34	19	31	41	33
Female	9	64	33	66	42	69	84	67
Total	14	100	50	100	61	100	125	100




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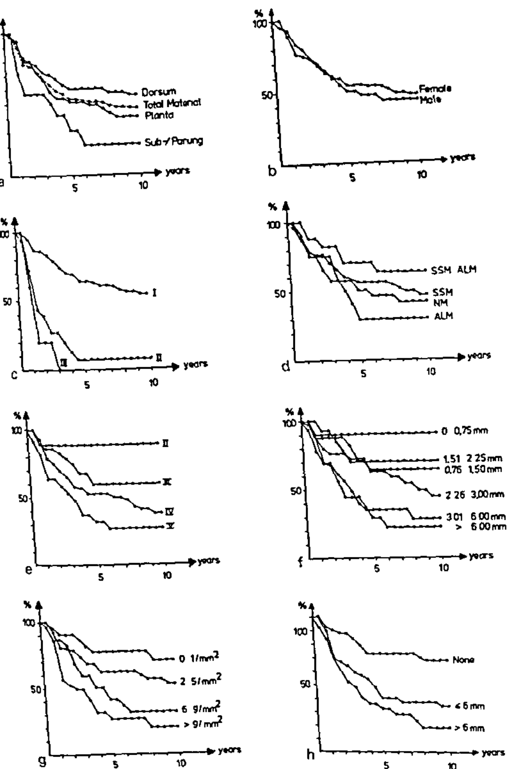



Fig. 5 Cumulative survival rates for patients with malignant melanoma of the foot according to (a) site of the foot (125 patients); (b) sex (125 patients); (c) clinical stage (125 patients); (d) type (120 patients); (e) level of invasion (121 patients); (f) tumour thickness (124 patients); (g) Mitotic rate (118 patients); and (h) ulceration (125 patients)

TABLE 2 *Clinical Stage of 125 Melanomas of the Foot Correlated to the Site of Tumour*

Clinical Stage	Sub /parung		Planta		Dorsum		Total	
	No	%	No	%	No	%	No	%
I	9	64	39	78	54	88	102	82
II	2	14	10	20	6	10	18	14
III	3	22	1	2	1	2	5	4
Total	14	100	50	100	61	100	125	100

Sex Of the 125 patients 84 were female and 41 male Table 1 demonstrates that the proportion of melanomas in each of the various regions of the foot was 2 1 women to men

The 10 year survival rates for women and men showed no significant difference ($X^2 = 0\ 094$ df 1 $P > 0\ 7$) (Fig 5b)

Clinical stage 102 patients were in stage I 18 in stage II and 5 in stage III Table 2 shows the regional distribution of lesions The 10 year survival was significantly higher for stage I patients (54%) than for stage II (7%) or III (0%) patients ($X^2 = 42\ 047$ df = 1 $P < 0\ 0005$) (Fig 5c)

Histological type Of 125 melanomas 55 were SSM 28 NM 20 ALM none LMM and 22 unclassifiable 17 of the unclassifiable melanomas histologically took up a position intermediate between SSM and ALM and were named SSM ALM A lentiginous proliferation of tumour cells was found as well as areas with moderate Pagetoid extension of tumour cells (Fig 3) In the remaining 5 melanomas of unclassifiable type it was uncertain whether or not the lateral intra-epidermal proliferation of tumour cells extended more than 3 rete ridges beyond areas of clear dermal invasion i e whether the melanomas were NM or melanomas with adjacent intra-epidermal component (ACM) (17) The 5 melanomas were designated NM ACM

As shown in Table 3 ALM and SSM ALM predominantly were localized sub /parungually and at planta The different types (SSM NM ALM and SSM ALM) did not influence the 10 year survival rate significantly ($X^2 = 3\ 061$ df 3 $P > 0\ 3$) (Fig 5d)

Level of invasion Of 121 melanomas 9 showed level II 29 level III 61 level IV and 22 level V In 2 patients polypoidal lesions level PM II were found One of these patients died of metastases 1 / years after operation while the other was still alive 7/ years after operation Further 2 melanomas were unclassifiable because of a very illdefined papillary reticular interface

Only one patient with a sub /parungual melanoma level II (and tumour thickness 0 64 mm) died of metastases half a year after operation Histologically total regression of the central part of the tumour was noted which was in agreement with

TABLE 3 *Histological Type of 125 Melanomas of the Foot Correlated to the Site of Tumour*

Histological Type	Sub /parung		Planta		Dorsum		Total	
	No	%	No	%	No	%	No	%
SSM	3	21	17	34	35	57	55	44
NM	4	29	7	14	17	28	28	22
ALM	5	36	12	24	3	5	20	16
SSM ALM	2	14	11	22	4	7	17	14
NM ACM	0	0	3	6	2	3	5	4
Total	14	100	50	100	61	100	125	100

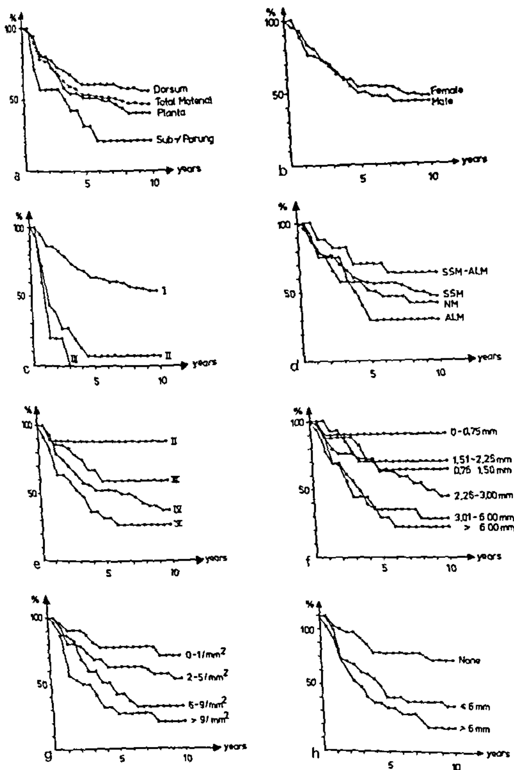


Fig. 5 Cumulative survival rates for patients with malignant melanoma of the foot according to (a) site of the foot (125 patients) (b) sex (125 patients) (c) clinical stage (125 patients) (d) type (120 patients) (e) *level of invasion (121* : (118 patients) and (h) ulceration (125 patients)

TABLE 4 *Level of Invasion of 125 Melanomas of the Foot Correlated to the Site of Tumour*

Level of Invasion	Sub /parung		Planta		Dorsum		Total	
	No	%	No	%	No	%	No	%
II	2	14	2	4	5	8	9	7
PM II	0	0	0	0	2	3	2	2
III	5	36	10	20	14	23	29	23
IV	3	21	29	58	29	48	61	48
V	4	29	9	18	9	15	22	18
Unclass	0	0	0	0	2	3	2	2
Total	14	100	50	100	61	100	125	100

TABLE 5 *Tumour Thickness of 125 Melanomas of the Foot Correlated to the Site of Tumour*

Tumour Thickness (mm)	Sub /parung		Planta		Dorsum		Total	
	No	%	No	%	No	%	No	%
0 –0 75	2	14	1	2	7	11	10	8
0 76–1 50	4	29	4	8	9	15	17	13
1 51–2 25	1	7	9	18	4	7	14	11
2 26–3 00	2	14	12	24	11	18	25	20
3 01–6 00	2	14	19	38	21	34	42	34
>6 00	3	22	5	10	8	13	16	13
Unclass	0	0	0	0	1	2	1	1
Total	14	100	50	100	61	100	125	100

TABLE 6 *Mitotic Rate of 125 Melanomas of the Foot Correlated to the Site of Tumour*

Mitoses/mm²	Sub /parung		Planta		Dorsum		Total	
	No	%	No	%	No	%	No	%
0–1	1	7	9	18	13	21	23	18
2–5	4	29	13	26	25	41	42	34
6–9	2	14	11	22	10	16	23	18
>9	3	21	15	30	12	20	30	24
Unclass	4	29	2	4	1	2	7	6
Total	14	100	50	100	61	100	125	100

TABLE 7 *Ulceration of 125 Melanomas of the Foot Correlated to the Site of Tumour*

Ulceration (mm)	Sub /parung		Planta		Dorsum		Total	
	No	%	No	%	No	%	No	%
0	3	21	20	40	35	58	58	46
≤6	6	43	20	40	16	26	42	34
>6	5	36	10	20	10	16	25	20
Total	14	100	50	100	61	100	125	100

information from the patient that the flat lesion originally had been elevated

10 year survival decreased significantly by deeper invasion Level II 89% level III 60% level IV 38% and level V 27% (T = 10 515 df 1 P <0 005) (Fig 5e)

Table 4 shows that the frequency of lesions level II and III was greater in sub-/parungual region (7/14 50%) than in the rest of the foot (31/107 29%), but the difference was not significant (X^2 2 541 df 1 P >0 1)

Tumour th ckness Of 124 melanomas 10 showed a thickness of 0–0 75 mm 17 of 0 76–1 50 mm 14 of 1.51–2 25 mm 25 of 2 26–3 00 mm 42 of 3 01–6 00 mm and 16 of >6 00 mm One melanoma was unclassifiable as it obviously had not been cut perpendicular to the original skin surface As described above only one melanoma less than 0 76 mm thick (and level II) with severe regression of the central part of the tumour matastasized causing the patient s death

The 10 year survival diminished significantly with increasing thickness 0–0 75 mm 90% 0 76–1 50 mm 64% 1 51–2 25 mm 70% 2 26–3 00 mm 44% 3 01–6 00 mm 28% and >6 00 mm 22% (T 16 109 df 1 P <0 0005) (Fig 5f)

Tabel 5 shows that tumours measuring less than 1 51 mm constituted a significantly greater propor tion of sub /parungual melanomas (6/14 43%) than of melanomas of the rest of the foot (21/110 19%), (X^2 4 118 df 1 P <0 05)

M tot c rate Of 118 lesions 23 showed 0–1 mitosis/mm² 42 2–5 mitoses/mm² 23 6–9 mitoses/mm² and 30 >9 mitoses/mm² 7 mela nomas were too small to permit examination of 1 mm² tumour tissue

The 10 year survival decreased significantly with increasing mitotic rate 0–1 mitosis/mm² 71% 2–5 mitoses/mm² 53% 6–9 mitoses/mm² 32% and more than 9 mitoses/mm² 20% (T – 15 982 df 1 P <0 0005) (Fig 5g)

Table 6 shows an equal mitotic activity in the various regions

Ulcerat on Of 125 melanomas 67 were ulcerated Ulceration was 6 mm or less in 42 lesions and grea[illegible]

[illegible] >6 mm (18%) (T – 19 118 df 1 P <0 0005) (Fig 5h)

Table 7 shows that 79% (11/14) of sub-/parungual melanomas were ulcerated as opposed to 60% (30/50) of planta and 42% (26/61) of dorsum

DISCUSSION

The present study comprised all patients operated on at the Finsen Institute for primary malignant melanoma of the foot in the period from 1949 to 1977 where the histological material was available and suitable for microscopic classification

Part of the material has previously been publis hed (19 20) As shown by Søndergaard (26) it is possible to evaluate the prognostic significance of histological type level of invasion and tumour thickness without serial blocking of the melanomas provided that at least one cross section through the most elevated part of the lesion is preserved for the histological examination As far as we know this requirement is fulfilled in the present material

Though the age of the patients varied from 22 to 94 years 64% (80/125) were older than 54 which is in accordance with the findings of *Petersen* (22) and *Magnus* (15)

11% (14/125) of the melanomas were located sub /parungually 40% (50/125) were of planta and 49% (61/125) of dorsum

The survival rate was significantly lower for patients with sub-/parungual melanomas than for patients with melanomas of the rest of the foot despite the fact that the sub-/parungual melanomas were not as thick or as deeply invading as the tumours of the planta and dorsum Melanoma in planta indicated a poorer prognosis than melanoma of the dorsum but the difference was not signifi [illegible]

[illegible] thought to be particularly high in toes and planta which may explain the very high mortality from melanomas of sub-/parungual region and planta

As was the case in other studies (9 11 15 16 19 22 27) we found more women than men in the material The proport on of [illegible]

[illegible] sexes was noted

82% of the patients were in clinical stage I which is more than expected This is explained by the greater frequency of insufficient histological material in patients stage II and III than in patients stage I At time of operation patients with sub / parungual lesions presented metastases twice [illegible]

[illegible] patients

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2–5	4	29	13	26	25	41	42	34
6–9	2	14	11	22	10	16	23	18
>9	3	21	15	30	12	20	30	24
Unclass	4	29	2	4	1	2	7	6
Total	14	100	50	100	61	100	125	100

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≤6	6	43	20	40	16	26	42	34
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SSM was the most prevalent type of melanoma (44%) of the foot followed by NM (22%) Both types were evenly distributed In accordance with other studies we found no LMM (6 13 27)

16% (20/125) of the tumours were readily classifiable as ALM according to the description of *Reed* (24) ALM was predominantly seen in planta and sub /parungually as reported by *Reed* (24) and *Clark et al* (6) According to *Clark et al* (6) ALM represents a clinicopathological entity However we found histologically a transition between SSM and ALM in that as many as 14% (17/125) of the melanomas (SSM ALM) showed characteristic features from both SSM and ALM This indicates that ALM is a subgroup of SSM rather than a specific entity which is further supported by the findings that prognosis is the same for patients with SSM as for patients with ALM

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As did others (7 9 12 14 16 18 25) we found that the survival rate diminished with increasing mitotic rate Although the presence of few mitoses is of importance for the diagnosis of malignant melanoma the prognosis was seriously aggravated only if more than 5 mitoses/mm^2 were noticed

Ulceration was seen in 54% of the tumours and was especially frequent in sub /parungual lesions 79% of which were ulcerated In accordance with other studies (1 7 9 12 14 25) we found that ulceration was correlated with a poor prognosis The greater the ulceration the lower the survival rate

CONCLUSIONS

1) Melanoma of the foot was predominantly seen in elderly patients as 80/125 patients were more than 54 years of age

2) The most prevalent type was SSM which occurred twice as often as NM No LMM of the foot was found One quarter of the melanomas of sub / parungual region and of planta were ALM but an equal number of melanomas histologically were intermediate between ALM and SSM indicating a close biological relationship between ALM and SSM The type of melanoma did not influence the survival rate significantly

3) The survival rate for patients with sub / parungual melanomas was significantly lower than for patients with melanomas of planta and dorsum

4) The proportion was 2 1 between women and men Sex did not influence the prognosis

5) As is the case with melanomas in other anatomical regions melanomas of the foot in clinical stage I were correlated with a better prognosis than melanomas in stages II and III

6) The following histological features indicated a bad prognosis Clark s level IV and V tumour thickness more than 2 25 mm more than 5 mitoses/mm^2 and presence of ulceration

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cta path microbiol scand Sect A 88 285–289 1980

TERATOGENIC EFFECT OF MATERNAL ALCOHOL CONSUMPTION ON THE MOUSE FETUS

A Histopathological Study

B BRUUN RASMUSSEN and N CHRISTENSEN

Department of Pathology Frederiksberg Hospital Copenhagen and Eye Pathology Institute Copenhagen Denmark

Rasmussen B Bruun & Christensen N Teratogenic effect of maternal alcohol consumption on the mouse fetus A Histopathological study Acta path microbiol scand Sect A *88* 285–289 1980

During pregnancy C3H mice were solely kept on a 10 or 20% liquid supply They had free access to solids All animals including a group of controls were chloroformed on the 19th day of pregnancy and the fetuses were examined for growth retardation intrauterine death and microscopic malformations Among the animals given alcohol we found a significantly raised incidence of malformations as well as a raised number of intrauterine deaths and significant growth retardation as compared to the control animals At the same time we found a clear dose-effect relation The malformations were localized to the facial skeleton the CNS the eyes and lungs

Key words Alcohol consumption maternal fetal death growth retardation microscopic malformations

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Accepted as submitted 8 ix 79

The fetal alcohol syndrome (FAS) was described as a well-defined syndrome by *Jones et al* (3–6) in children born by mothers with a history of heavy alcohol consumption before and during pregnancy The syndrome consists of general growth retardation defects in the facial skeleton and CNS and as a less constant phenomenon heart malformations and joint deformities Several reports have later confirmed the teratogenic effect of alcohol in man (2 7 8 9 13 15)

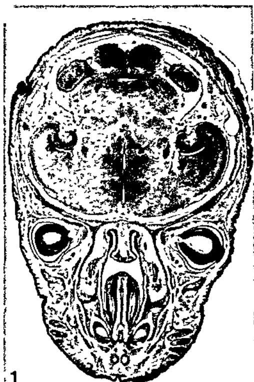
A lot of experimental work has been published especially on rodents (1 10 11 12 16) In the [illegible] and in *Chernoff's* work (1) one finds changes in mice comparable to those found in humans

Up till now all the reports have confined themselves to a mascroscopic and stereomicroscopic examination of the animal fetuses In our study we have used a microscopic screening technique and at the same time looked for a dose-effect relation by giving the animals different alcohol concentrations

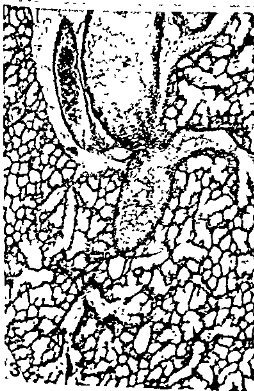
MATERIAL AND METHODS

The [illegible]

[illegible] – one mouse in each cabin One group of mice – the control group – had free access to food and


1


2


3


4

water In two experimental groups the only liquid offered throughout pregnancy was in one group 10% alcohol in the other 20% while they had free access to solids The amount of consumed water and alcohol solution was determined by daily weight control of the bottles

The control group consisted of 131 fetuses the group given 10% alcohol 72 fetuses and the group given a 20% solution 66 fetuses On day 19 of pregnancy all mice were chloroformed and the fetuses removed by sectio The number of intrauterine deaths were registered as well as the weight of the fetuses A stereomicroscopic examination was then performed For the histological screening the fetuses were decapitated and the heads fixed in Bouin s solution for 4 days mounted in paraffin and cut in serial sections The bodies too were fixed in Bouin s solution and after 4 days of fixation by a horizontal transverse section separated in two at the level

hematoxylin eosin and ad modum Van Gieson – Hansen combined with alcian blue All the slides were then screened for microscopic malformations

The Kruskal Wallis one way analysis of variance was used to evaluate differences in weight and concerning fetal death and number of malformed fetuses the chi square test was employed

RESULTS

During the experiment none of the animals show signs of disease and the behavior of the anim drinking alcohol did not differ from that of control group

Measurements of the daily alcohol consumpt showed in both experimental groups rising val throughout pregnancy and the initial minir liquid intake was always higher than the minir daily requirement of the animals The aver amount of consumed alcohol throughout pregnar was for animals given a 10% solution 83 5 (range 73 2–90 6) and for the animals given a 20 solution 56 7 ml (range 52 0–78 0) The to amount of alcohol was thus larger in the gro given 20% (11 3 gr) than in the group given 10 (8 3 gr) The reproductive data are given in Table which shows the number of pregnant and no pregnant mice the number of living fetuses and t resorption incidence given as the number intrauterine deaths as a percentage of the tot number of implantations Furthermore the tab shows the mean birth weight of the fetuses

Of the 46 expected pregnant mice in the materia 9 were shown not to be pregnant at day 19 in spi

TABLE 1 *Pregnant and Non Pregnant Mothers Live Fetuses Resorption Incidence and Mean Birth Weght*

	Pregnant	Non pregnant	Live fetuses	Res %	Weight (mean gms
Control	17	0	131	7 1	10 1 (8 7–12 0)
10%	10	1	72	8 9	9 3 (8 3–10 0)
20%	10	8	66	21 4	7 7 (5 7–9 6)

TABLE 2 *Number of Live Fetuses Number of Malformed Fetuses and Number of Defects in the Face Cerebrum Eyes and Lungs*

	Live	Malformed	Face	Cerebrum	Eyes	Lungs
Control	131	4	1	3	2	0
10%	72	11	0	9	3	5
20%	66	48	26	27	19	28

Fig 1 Horizontal section through the normal mouse head on the 19 day of gestation (13x)

Fig 2 Horizontal section through the head of treated fetus on day 19 of gestation showing microcefali and open eyelids (13x)

Fig 3 Section showing normal lung of fetus on the 19 day of gestation (100x)

Fig 4 Section of lung from treated animal on day 19 of gestation showing severe hypoplasia Same magnification as Fig 3 (100x)

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5 *Jones K L et al* Outcome in offspring chronic alcoholic women Lancet *I* 1076–1078 1974
6 *Jones K L & Smith D W* The fetal alcohol syndrome Teratology *12* 1 10 1975
7 *Little Ruth E* Moderate alcohol use during pregnancy and decreased infant birth weight Am J Public Health *67* 1154–1156 1977
8 *Mulvihill J J & Yeager A M* The fetal alcohol syndrome Teratology *13* 345–348 1976
9 *Palmer R H et al* Congenital malformations in offspring of a chronic alcoholic mother Pediatrics *53* 490–494 1974
10 *Papara Nicholson D & Telford I R* Effect of alcohol on reproduction and fetal development in the guinea pig Anat. Rec. *127* 438 1957
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13 *Streissguth A P* Fetal alcohol syndrome An epidemiologic perspective Am J Epidemiol *107* 467–478 1978
14 *Streissguth A P* Personal communication 1977
15 *Tenbrinck M S & Buchin S Y* Fetal alcohol syndrome Report of a case J Am Med Ass *232* 1144 1975
16 *Tze W J & Lee M* Adverse effects of maternal alcohol consumption on pregnancy and fetal growth in rats Nature *257* 479–480 1975

of an obvious vaginal plug 8 of these were found in the group given 20% alcohol and 1 in the group with 10% None of the non pregnant uteri showed any histologic sign of implantation

The resorption incidence in the experimental groups was significantly raised as compared to that of the control group ($p < 0\ 05$ and $0\ 01$) and it was greater in the group given a 20% solution than in the 10% group ($p < 0\ 05$) The growth retardation expressed as the mean birth weight was significant in both experimental groups compared to the control group and again there was a significant difference between the two experimental groups ($p < 0\ 0005$) Table 2 gives the number of living fetuses the number of malformed fetuses and the number of the malformations most frequently shown

The frequency of malformed fetuses was significantly raised in the experimental groups compared to the control group ($p < 0\ 01$ and $0\ 001$) and there was a significant number of malformed fetuses in the group given 20% as compared to the group given a 10% solution ($p < 0\ 001$) As shown in the figures representing the different types of malformations several of the fetuses had more than one malformation

Pathology

At the stereomicroscopic examination a certain pattern of malformations was found all located to the head In the group given a 20% solution there were certain characteristics a shortening of the nose sometimes in combination with low set ears and open eyes The eye defect also occurred isolated in a few animals in the control group There were no malformations of extremities no abdominal wall defects and no defects of the neural tube The microscopic defects as the streomicroscopic examination had suggested were mostly localized to the head

Fig 1 shows a horizontal cut through the head of an animal from the control group In fetuses with shortened nose a uniform hypoplasia of all structures in the splanchnocranium and its soft parts was found often combined with a moderate microcephalia and dilated ventricles of the brain Severe forms of microcephalia were often combined with open eyes (Fig 2) The only consistent change in the body was varying degrees of isolated hypoplasia of the lungs a change that especially occurred among animals given a 20% solution Fig 3 shows a horizontal cut of the lung from an animal in the control group while Fig 4 – same magnification – shows lung tissue from an animal in the experimental group In the lungs most heavily affected narrow bronchioli with a folded mucosa and only here and there a few small alveolae like structures covered by columnar epithelium were found

DISCUSSION AND CONCLUSION

The amount of consumed alcohol was decided by daily weighing of the bottles and all bottles were before use controlled for spontaneous loss of content The amount of consumed food was not registered since the amount of alcohol here used does not influence the calorie intake from other food sources (1 16)

The occurrence of 8 non pregnant mother animals in the group given a 20% solution cannot be explained by the unreliability of the method of confirming pregnancy As microscopy of these uteri revealed no histologic sign of earlier or present pregnancy it is likely that the alcohol has interfered with implantation in these animals We have n t seen this phenomenon described in other experimental models

Although several experimental reports on the teratogenic effect of alcohol are available a combination of malformations that are comparable to FAS in man was first described by *Chernoff* (1977) He found apart from perinatal growth reduction and incomplete ossification malformations in the CNS the skeleton and the heart Like Chernoff we also found dilated ventricles of the brain and eyelid defects but we did not find aplasia of the corpora callosum We did not find heart malformations e g septal defects The reason for this is probably that our method of cutting horizontal sections of the body would not disclose small defects but only bigger ones The difference in results concerning malformations of the skeletal system can be explained by Chernoff making special preparations regarding this part of the body We have not in earlier experimental studies seen descriptions of the pulmonary changes neither have they been described in children with FAS (14)

Even though only two alcohol concentrations have been examined there was a clear dose-effect relation This was seen not only in regard to growth reduction resorption incidence and the frequency of malformations but also in the fact that the high alcohol concentration apparently can interfere with nidation On the other hand there is no morphological difference between malformations induced by the various alcohol concentrations

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The authors wish to acknowledge the assistance of *Kurt Jensen and Eggert Hansen* laboratory technicians *P ul Schl cht ng* M D and *S Olesen Larsen* Statistician Statens lægevidenskabelige Forskningsråd

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Acta path microbiol scand Sect A 88 291-297, 1980

SURFACE CHANGES IN THE ARTICULAR CARTILAGE OF RABBIT KNEE DURING IMMOBILIZATION. A SCANNING ELECTRON MICROSCOPIC STUDY OF EXPERIMENTAL OSTEOARTHRITIS

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Research Laboratory of the Invalid Foundation and Institute of Occupational Health Helsinki Finland

Candolin T & Videman T Surface changes in the articular cartilage of rabbit knee during immobilization A scanning electron microscopic study of experimental osteoarthritis Acta path microbiol scand Sect A 88 291-297 1980

Osteoarthritis was produced by the immobilization of rabbit knees in extension for 1-8 weeks (with a subsequent mobilization period of 0 or 8 weeks) The development of articular surface changes in the tibia the femur and the patella was examined with scanning electron microscopy (SEM) During the development of osteoarthritis the normal undulations and fine regular fibre network disappeared the number of fibres and the variation in the thickness of the fibre bundles increased and scaly irregularities appeared Some degenerative changes in the contralateral non [illegible]
appeared The norma [illegible]
femoral condyles Th [illegible]
of earlier studies but [illegible]
the histological changes The SEM method seems to be useful for the study of articular surfaces however the area studied must be defined

Key words Cartilage immobilization osteoarthritis scanning electron microscopy

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Received 22 x 79 Accepted 7 iii 80

Scanning electron microscopy (SEM) presents a unique method for studying joint surfaces (*Redler & Zimny* 1970) Generally the methods used for examining normal and osteoarthritic joints provide a limited view of the condition of joints Studies on [illegible]

[illegible] dimensional sections The characteristics of osteoarthritis are revealed best by macroscopic and/or SEM examinations However the results of SEM studies of normal articular cartilage are somewhat contradictory and there is doubt as to whether or not some of the changes noted are artefacts (*Ghadially et al* 1977)

The surface of normal human articular cartilage is gently undulated and consists of an arrangement of regular ridges (1-100 μm) and bowl shaped depressions, sometimes forming »figure eights« The articular surface is composed of a dense random network of fine collagen fibrils (0 1 μ) (*Clarke* 1971, *Cotta* 1973 *Redler* 1974 [illegible] 1969) Age [illegible]
articular ca [illegible]
depressions increasing and the number of ridges decreasing with age (*Inoue* 1972)

Osteoarthritic articular surfaces are disrupted and exhibit irregular nodular fibres and microcraters (*Redler* 1974 *Refior* 1074) The degeneration of the surface of articular cartilage has been classified primarily on the basis of surface roughness into six

stages by *Kotani et al* (1975) In transmission electron microscopy osteoarthritic cartilage shows increasing forms of surface folding and splitting with debris and collagen fibres of diminished size and an altered structure (*Weiss* 1973)

On the other hand the SEM features of an arthrotic joint vary greatly from one kind of joint to another and from one site to another, and it is difficult to distinguish different stages of the arthrotic process when specimens are taken from a big human joint (*Weiss* 1973 *Kotani et al* 1975, *Inoue* 1972)

If the changes are compared with those of a non-immobilized leg and the legs of control rabbits the possible methodological errors should be accounted for

In the present study we have examined articular surface changes of experimental osteoarthritis produced by immobilization using SEM The development of this condition has been studied earlier radiographically histologically and biochemically as a function of immobilization time (*Langenskiöld et al* 1979) The aim of the present study was to correlate the changes found with SEM with the results of earlier experiments on the development of osteoarthritis

MATERIAL AND METHODS

In a pilot study we used 8 rabbits to determine suitable and easily definable regions for examination

In the present study we used 24 9 month-old rabbits of both sexes Six were immobilized for 1 to 2 weeks (*Group II*) and 6 from 6 to 8 weeks (*Group III*) without a subsequent mobilization period Six rabbits immobilized for 3 to 5 weeks were mobilized for more than 8 weeks after the immobilization (Group IV) the last 6 rabbits were controls living normally in their coops (Group I)

Osteoarthritis was produced in one knee of the rabbits with an earlier published immobilization method (right knee held in extension with a PVC plastic splint and a Tensoplast® bandage) (*Langenskiold et al* 1979)

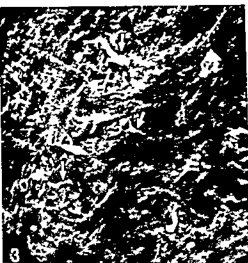
Immediately after the rabbits were killed the following samples were carefully prepared the patella the femoral condyles and the lateral tibial condyles including the whole thickness of cartilage with a 5 mm thick piece of subcondral bone The samples were washed in physiological saline and fixed in a 2 5% glutaraldehyde solution buffered by 0 1 M cacodylate for 2–7 days at + 4 °C The samples were thereafter washed in a solution of 0 1 M cacodylate and 0 2 M sucrose and dehydrated by a graded series of increasing ethanol concentrations for 3 days Most of the samples were dried in air i e all except the patellae of the 6 control rabbits which were dried according to the critical point methos (*Cameron et al* 1976) The samples were



Group I control rabbits living normally in their coops
Group II the right leg immobilized (IZ) for 1–2 weeks and followed (F) for 0 weeks
Group III IZ = 6–8 weeks F = 0 weeks
Group IV IZ = 3–5 weeks F = 12 weeks

Fig 1 Articular cartilage of *medial femoral condyle* The picture represents a control rabbit (Group I) and shows regular undulations and no fibre bundles (700 ×)

Fig 2 Articular cartilage from the *medial femoral condyle* of a rabbit from Group II The picture represents changes in the immobilized knee the articular surface shows fibre bundles of varying thicknesses (arrows) but the undulations are clearly visible (700 ×)



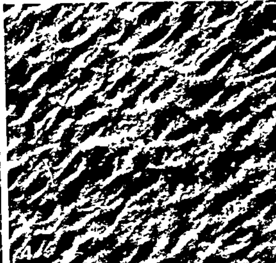


Fig 3 Medial femoral condyle of a rabbit from Group III The picture shows the appearance of the immobilized knee there is variation in the fibre bundles scaly irregularities (arrows) and a disappearance of regular undulations (700 ×)

Fig 4 The picture shows the corresponding region from the contralateral femur of the same rabbit as in figure 3, the appearance is normal (700 ×)

thereafter attached to a specimen holder and coated with a layer of carbon and gold in a vacuum evaporator The JEOL JSM U scanning electron microscope of the Department of Electron M[illegible]

[illegible]

area of the femoral condyle against the tibia (in extension)

In the SEM examination the specimens were tilted at an angle of 30–40° All samples were photographed at a magnification of 50 × and the central point was photographed with magnifications of 500 and 1500 × Two workers assessed the photographs independently and they evaluated every specimen separately with respect to fibre appearance and scaly irregularities (sharp folds) using the following scale 0 = no degenerative changes 2 – slight changes 4 – moderate changes and 6 = severe degenerative changes (*Kotani et al* 1975)

RESULTS

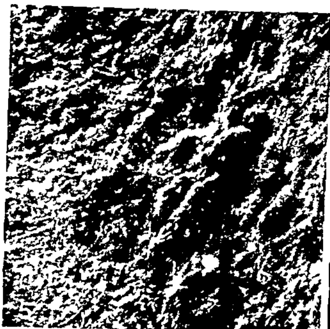
The appearance of articular surfaces varies in different joints and also in the different regions of the same joint and therefore only corresponding areas were compared

The normal appearance of the central region of the articular surface of the medial femoral condyle showed regular ridges and undulations (Fig 1) These features disappeared during immobilization In addition the fibre bundles became thicker and varied in thickness, and some scaly irregularities also appeared (Figs 2–6) There was considerable normal variation but statistically the degenerative changes increased clearly with increasing immobilization time, and the contralateral surface remained normal (Fig 7)

The normal weight bearing area of the articular surface of the lateral tibial condyle showed smooth undulations with or without a fine regular network (Fig 9) During immobilization the undulations became shallower and irregular fibre bundles appeared The surface also became scaly (Fig 8) The aforementioned degenerative changes increased as the immobilization period increased and the appearance on the contralateral surface remained normal (Fig 10)

The normal view of the central region of the articular surface of the patella consisted of weak undulations and a fine fibre network, and the general appearance was regular (Fig 11–12) An increase in the [illegible]

[illegible] of irregularities in the articular surface of the non-immobilized knees in correlation to the length of the immobilization period was not statistically significant (Fig 13–14) It was also obvious that degenerative changes progressed after the immobilization period The degenerative changes were »slight to moderate« after the shortest immobilization time used (= 1 week)



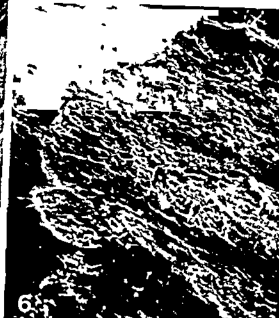


Fig 5 Medial femoral condyle of a rabbit from group IV The picture shows the surface changes from immobilized knee ridges vary and the surface is irregular (700 ×)

Fig 6 Medial femoral condyle The picture (200 ×) shows severe osteoarthritic changes in the central part (A) of picture almost all of the articular cartilage has disappeared The knee was immobilized for 8 weeks before the ra[illegible] was killed (Group III)

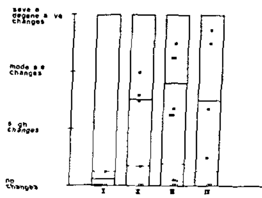


Fig 7 Assessments of the degenerative changes found with SEM of the articular cartilage surfaces of the medial femoral condyles ■ assessments of the SEM photo graphs of the immobilized leg — median for the leg assessments □ assessments of the SEM photographs of the nonimmobilized leg median for the nonimmobi lized leg assessments

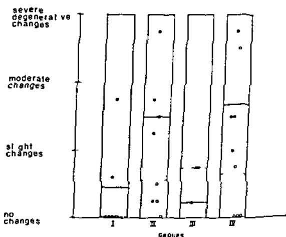


*Fig 10 Assessment of degenerative changes found wi*t SEM of the articular cartilage surfaces of the lateral tibia condyles

Fig 8 Lateral tibial condyle of a rabbit from Group IV The picture shows the arthrotic changes from the immobilized knee there are fibre bundles of varying thicknesses scaly irregularities and a partial disappearance of the undulations (700 ×)

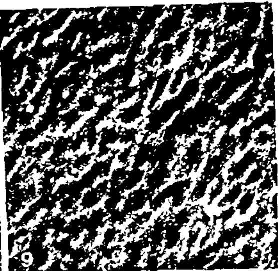
Fig 9 The photograph is of the corresponding region of the contralateral non immobilized knee of the same rabbit as in figure 8 the appearance is normal (700 ×)

Fig 11–12 Articular cartilage of the *patella* The pictures represent Group I and show normal variations Fig 11 reveals weak undulations and in fig 12 the fine fibre network is dominant (700 ×)

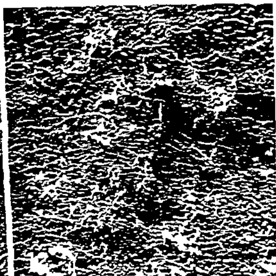
Fig 13 Articular cartilage of the *patella* The picture represents changes in Group III Irregular fibre bundles are the predominant feature in this stage of osteoarthritis development (700 ×)

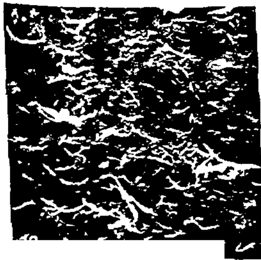
Fig 14 Articular cartilage of the *patella form group IV The picture represents severe osteoarthritic changes* (700 ×) The inset shows the appearance from the non immobilized knee of the same rabbit and there are also some degeneration changes in this picture (200 ×)

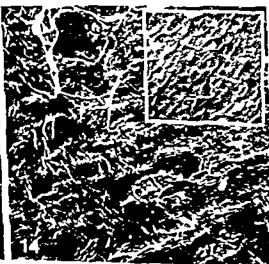

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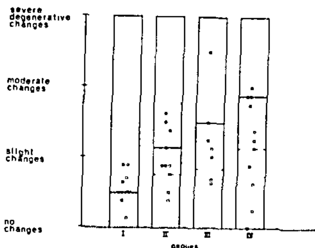


Fig 15 Comparison of the assessments of the degenerative changes found with SEM of the articular cartilage of the patella

The irregularities were more marked in the rabbits immobilized 3–4 weeks and followed for 8 weeks than in rabbits with only an immobilization period of 6–8 weeks (Fig 15)

DISCUSSION

There is some reason to believe that compression against a joint surface increases during immobilization causing arthrotic changes in the joint (*Videman & Michelsson* 1977) If this assumption is correct, the degenerative changes in the central region of the articular cartilage occur first and the marked changes in the marginal part of the joint (e g osteophytes) follow Therefore we examined the load bearing area of articular cartilage in the present study

There was no general difference between the surfaces of the samples dried with either the critical point technique or air Both methods gave an appearance of weak undulations and a fine fibrillar network In the specimens from tibial condyles there were often fissures which were thought to be artefacts The fissures were caused in our opinion by the different contractile forces in the bone and cartilage during the drying and it can be assumed that the loading resistance in tibial condyles is good against compressive forces and weak in the tangential direction Neither is a fibre network a general feature of tibial condyle surfaces

The earliest osteoarthritic changes in the radiographs of rabbit knees are visible after 2 weeks of immobilization, and these changes are definite after 3–4 weeks The first histological arthrotic cartilage changes are seen after 10 days of immobilization and with biochemical methods it is noted that the glycosaminoglycan synthesis rate increases after 6 days of immobilization After 5–6 weeks of immobilization rabbit knees show moderate or severe osteoarthritic changes, including a disappearance of articular cartilage (*Langenskiold et al* 1979, *Eronen et al* 1978)

The SEM changes that occurred during immobilization correlated well with corresponding histological changes After 1 week of immobilization the degenerative SEM changes were relatively more *marked than those found in a histological examination* This difference can be easily understood if it is remembered that osteoarthritic changes start in cartilage as fibrillation of the surface (*Sokoloff* 1969, *Weiss* 1973)

Typical features of the articular surface from tibial and femoral condyles with developing osteoarthritis are the increasing number of fibres and the varying thicknesses of the fibre bundles However, on the articular surface of the patella a fibre network is a normal characteristic

In earlier studies with experimental immobilization osteoarthritis the contralateral non immobilized knee has been identical when compared with the knees of rabbits living normally in their coops (*Eronen et al* 1978, *Langenskiold et al* 1979) In *the present study the articular surface of the patella* on the non immobilized side showed some degenerative changes after the immobilization The changes were slight but the variation was relatively great Not many animals were examined and the difference was not evaluated statistically (Fig 15) It is possible that the immobilization of one knee strains the other or that there are some general factors in the development of osteoarthritis which affect all joints

This study was supported by grants from the *Sigrid Juselius Foundation* and *Juho Vainio Foundation*

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Acta path microbiol scand Sect A 88 299–306 1980

IMMUNOHISTOCHEMICAL DEMONSTRATION OF ALPHA-1-ANTITRYPSIN IN LIVER TISSUE

A Methodological Investigation

PER PRÆTORIUS CLAUSEN

Department of Pathology Herlev Hospital and Department of Pathology Hvidovre Hospital University of Copenhagen

Clausen Per Prætorius Immunohistochemical demonstration of alpha 1 antitrypsin in liver tissue A methodological investigation Acta path microbiol scand Sect A 88 299–306 1980

The influence of the tissue preparation on the immunohistochemical demonstration of alpha 1 antitrypsin (AAT) in liver tissue was evaluated using autopsy and biopsy material with and without AAT globules On comparing frozen sections of unfixed material with paraffin sections of formalin fixed material a slightly better preservation of the immunoreactivity of AAT was observed in frozen sections On comparing different fixatives fixation in 10% neutral buffered formalin Lillie s AAF or 96% ethanol/1% acetic acid caused a clearly better preservation of demonstrable AAT than fixation in Clarke s or Bouin s fixatives The fixation time had only minor influence when using fixation times within a week Extremely long fixation for several months caused however a clear reduction in demonstrable AAT Pre [illegible] tissue caused an increas[illegible] time On comparing [illegible] peroxidase/anti perox[illegible] sensitivity was observed between the three techniques It is concluded that for the immunohistochemical demonstration of AAT in liver tissue the employment of indirect immunoperoxidase staining on formalin fixed paraffin embedded material is recommendable as it combines a sensitive staining technique with a satisfactory preservation of immunoreactivity and tissue morphology

Key words Alpha 1 antitrypsin liver tissue immunohistochemistry fixation

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In 1971 *Sharp* described the presence of PAS positive diastase resistant globules in liver tissue from patients with alpha 1 antitrypsin (AAT) deficiency of Pi phenotype ZZ (24) Using immunohistochemical techniques it was shown that these globules contained AAT and the globules are therefore usually named AAT globules

In a number of papers AAT globules have later on been described in as well ZZ as MZ individuals in both children and adults with and without liver disease (1 4 5 6 8 12 16 17)

The employment of immunohistochemical staining for AAT in liver tissue as a supplement to the PAS/diastase staining is important among others because PAS positive diastase resistant globules with negative reaction for AAT have been described in individuals with the phenotype MM (20 25) In addition heterozygous (MZ) individuals in some cases lack PAS positive diastase resistant globules while AAT deposits still can be demonstrated in the liver tissue (14)

The possibility of identifying an individual as a carrier of the Z gene by using immunohistochemical staining for AAT of the liver tissue has special importance in connection with retrospective investi

gations made on autopsy and biopsy material where analysis of the serum cannot be performed Furthermore the immunohistochemical staining technique is valuable because AAT phenotyping of serum until now only has been performed in a few specialized laboratories while *immunohistochemi* cal investigations on routine processed material in increasing degree are used in histopathological diagnostics

A prerequisite for using immunohistochemical investigation for AAT is however that the staining results are reproducible and that *the preparation of* the tissue does not significantly affect the staining result In previous papers discrepancies as regards the suitability of different forms of tissue *prepara* tion and immunological techniques for demonstra tion of AAT in liver tissue have been described (3 15 18 25)

The present investigation aims at examining the *influence* of the tissue preparation on the immuno histochemical demonstration of AAT in liver tissue and to compare results obtained by using three *different immunohistochemical* techniques

MATERIAL AND METHODS

Tissue and Tissue Preparation

The material consists of liver tissue *from 16 different* patients In six cases (A–F) the tissue was obtained in connection *with autopsy* performed within 12 hours after death in one case (G) obtained peroperatively as a surgical knife biopsy and in the remaining 9 cases (H N) as percutaneous needle biopsies A survey of the tissue materials chief histological diagnoses fixatives and *fixation times used is shown in Table 1*

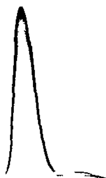
Autopsy material was divided in pieces measuring 20 × 20 × 5 mm Liver tissue from A–C contained intracytoplasmic PAS positive diastase resistant globules with positive reaction for AAT and measuring up to at least 3 µm Liver tissue *from D–F did not conta n PAS* positive globules

The autopsy material was immediately after removal fixed at 20 °C in the following fixatives 10% phosphate buffered formalin pH 7 Lillie s AAF Clarke s fixative and Bouin s fixative (13 a) In each fixative the tissue was incubated for 1 4 8 and 24 hours and 7 days In a few cases *tissue was in addition fixed in 10% buff red* formalin for 18 months Following fixation the tissue was dehydrated in ethanol and xylene and embedded in

TABLE 1 *Source of Tissue Diagnosis and Fixat on*

Tissue		Chief histological diagnosis	Fixatives					Fixation time						
			Buffered formalin	AAF	Clarke	Bouin	EA (4 °C)	1 hour	4 hours	8 hours	24 hours	7 days	24 days	18 months
Autopsy with AAT globules	A	Chronic congestion	+	+	+	+	+	+	+	+	+	+	+	+
	B	Portal fibrosis	+	+	+	+	+	+	+	+	+	+	+	+
	C	Macronodular cirrhosis	+								+	+		
Autopsy without AAT globules	D	Chronic congestion	+	+	+	+	+		+		+	+		
	E	Normal	+	+	+	+	+		+		+	+		
	F	Normal	+	+	+	+	+		+		+	+		
Biopsy with AAT globules	G	Macronodular cirrhosis	+						+					
	H	Non specific changes	+						+					
	I	Large duct obstruction	+						+					
	K	Hepatocellular carcinoma	+						+					
	L	Micronodular cirrhosis	+						+					
Biopsy without AAT globules	M	Macronodular cirrhosis	+						+					
	N	Normal	+						+					
	O	Micronodular cirrhosis	+						+					
	P	Non specific changes	+						+					
	Q	Large duct obstruction	+						+					

EA *96% ethanol* + 1% acetic acid



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Fig 1 Test of the monospecificity of anti alpha 1 antitrypsin by crossed immunoelectrophoresis 2 µl of 25 % pooled normal human serum has been run against anti alpha 1 antitrypsin at a concentration of 2 µl/cm² gel



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Fig 2 Test of the purity of alpha 1 antitrypsin by crossed immunoelectrophoresis 2 µl alpha 1 antitrypsin (1 mg/ml) has been run against anti human serum at a concentration of 8 µl/cm² gel

Paraplast at 59 °C In addition to the fixatives mentioned tissue from A and B was fixed at 4 °C in 96% ethanol and 1% acetic acid (EA) for 4 8 and 24 hours and 24 days In these cases the tissue was dehydrated in ethanol and xylene at 4 °C increasing to room temperature and embedded in Paraplast at 56 °C (22)

Additional tissue from A and B fixed in buffered formalin for 18 months was washed for 48 hours in 0 1 M phosphate buffer pH 7 5 at 4 °C frozen with CO_2 ice and stored at −20 °C Some tissue from C–F was frozen unfixed at −20 °C

The biopsies with AAT globules (G–L) and without AAT globules (M–Q) were fixed at room temperature for 24 hours in 10% phosphate buffered formalin pH 7 dehydrated in ethanol and xylene and embedded at 59 °C Following embedding or freezing serial sections at 5 µm were cut and placed on gelatinized slides

Pretreatment of Sections with Proteolytic Enzymes

Deparaffinized sections from some of the formalin fixed material (B) were before the staining incubated at 37 °C in 0 1% trypsin (SIGMA type no III) or 0 1% protease (SIGMA type no V) for 15 min in 0 0045 M calcium chloride adjusted to pH 7 8 with 0 1 M sodium hydroxide (10 11)

Antibodies and Control Reagents

Antibody no 1 Anti human AAT (immunoglobulin (Ig) fraction of rabbit antiserum) DAKO Denmark code no 10 012 lot 017 118 and 029 A protein concentration respectively 11 1 10 6 and 10 6 g/l Titre 200 (expressed as the amount (microgram) of antigen which has to be added to 1 ml antibody in liquid phase in order to obtain a supernatant without demonstrable amounts of antigen or antibody (23)) Anti AAT was used in various dilutions (see below)

The monospecificity of anti AAT was tested in crossed immunoelectrophoresis against normal human plasma Only one precipitation line appeared (Fig 1) The proof of the reaction with AAT and not with other serum proteins was demonstrated in crossed immunoelectrophoresis against human AAT

Antibody no 2 Anti rabbit immunoglobulin (Ig fraction of swine antiserum) DAKO code no 21 090 lot 078 E protein concentration 21 g/l titre 400 The antibody was used in dilution 1 10 determined by titration

Antibody no 3 Anti rabb

immunoglobulin molecule in average 0 8 peroxidase molecule is conjugated) titre 19 The conjugate is produced using the two step glutaraldehyde method (2) Working dilution 1 20 was determined by chess board titration.

Antibody no 4 Complexes of horseradish peroxidase/rabbit anti horseradish peroxidase (PAP) DAKO code no Z 113 lot 049 antibody concentration 1 98 g/l peroxidase concentration 0 75 g/l molar peroxidase/antibody ratio in average 1 5 Working dilution 1 50 determined by chess board titration

Antibody no 5 Anti rabb

titre 100 Working dilution 1 20 determined by chess board titration

For control stainings the following reagents were used

Control no 1 Anti human AAT (antibody no 1) absorbed with human AAT The purity of the AAT was controlled in crossed immunoelectrophoresis against

anti human serum One strong and one much weaker precipitation line appeared and AAT was therefore based on this criterion almost completely pure (Fig 2) The amount of AAT necessary for the absorption of anti AAT was determined by *Sewell* titration (23) The antibody was subsequently absorbed with AAT used in 25% excess The mixture was stored for 1 hour at 25 °C and 48 hours at 4 °C (interrupted by regular shaking for 5 min every second hour) and at last the mixture was centrifuged The supernatant – the absorbed antiserum – did not react with human serum in crossed immunoelec trophoresis The absorbed antiserum was used in the same dilutions as used with non absorbed antiserum

Control no 2 Ig fraction of serum from non immunized rabbits DAKO code no X 903 lot 076 Protein concentration 20 g/l Used in dilution 1 80

Control no 3 Swine serum from non immunized swine Used undiluted

Immunohistochemical Techniques

All staining reactions were performed at room temperature and the following techniques were used

I Indirect immunoperoxidase technique (IP) The staining procedure comprises the following steps

1) Methanol 0 5% H_2O_2 30 min (only when using fixed material)
2) 10% swine serum in phosphate buffered saline (PBS) pH 7 2 15 min
3) Rabbit anti human AAT variable dilution 30 min
4) Peroxidase conjugated swine anti rabbit immunoglo bulin 1 20 30 min
5) 0 04% 3 amino 9 ethylcarbazole (SIGMA) in di methylformamide (9) 0 01% H_2O_2 in 0 5 M sodium acetate/acetic acid buffer pH 5 (15 min)
6) Mounting in Apathy gum syrup (13 b)

Control stainings were performed by replacing the reagents one at a time in the following manner a) step 3 incubation with PBS b) step 3 incubation with control no 1 c) step 3 incubation with control no 2 and d) step 3 + 4 incubation with PBS

II Peroxidase anti peroxidase technique (PAP technique) Step 1 2 and 3 performed as described for IP technique

4) Swine anti rabbit immunoglobulin 1 10 30 min
5) PAP 1 50 30 min

Step 6 and 7) Staining and mounting as described in IP technique step 5 and 6

Control stainings were performed as described in method I In addition the following controls were performed a) step 4 Incubation with control no 3 b) Step 3–5 incubation with PBS

In technique I and II all antisera were diluted in 10% swine serum in PBS Between each step sections were washed in PBS for 3 × 5 min

III Indirect immunofluorescence technique (IF)

1) PBS 5 min
2) Rabbit anti human AAT variable dilution 30 min
3) FITC-conjugated swine anti rabbit immunoglobulin 1 20 30 min
4) Mounting in glycerol tris buffer pH 8 5

Control stainings were performed by replacing the reagents one at a time in the following manner a) step 2 incubation with PBS b) step 2 incubation with control no 1 and c) step 2 incubation with control no 2 All antisera were diluted in PBS Wash between each step with PBS for 3 × 5 min

Evaluation

The staining results of IP and PAP techniques were evaluated using a DIA LUX light microscope Leitz eye piece 10× objective 40× N A 0 65 IF technique was evaluated using Leitz universal fluorescent microscope with transmitted light and Dark Field Ultra Condenser N A 1 2 and objective 40× 0 75 N A eye piece 10× Light source HBO 200 W/4 pressure mercury lamp Filters Primary interference filter and secondary 3 mm glass filter matched with the primary filter (21)

All tissue was incubated in two fold serial dilutions of anti AAT serum The evaluation of the influence of fixative fixation time paraffin embedding pretreatment with proteolytic enzymes and immunohistochemical technique used on the staining results was based on a comparison of the highest dilution of anti AAT which still gave a clear positive reaction (end point titre) For each parameter examined all sections were stained on the same day and double staining series were performed independently The variation between end point titres of two comparable staining series was at most one dilution step The proportion between end point titres within each staining series was constant

TABLE 3 *Influence of Fixative and Fixation on the Immunolog*

TISSUE	10% buffered formalin			Lillie s AAF			
	4 h	24 h	7 days	4 h	24 h	7 days	4 h
A	1 12800	1 12800	1 12800	1 12800	1 6400	1 6400	1 16
B	1 25600	1 12800	1 12800	1 51200	1 12800	1 12800	1 64
C		1 25600	1 25600				

The table shows the highest dilution of anti alpha 1 antitrypsin which s

TABLE 2 *Influence of Tissue Preparation on the Immunological Reactivity of Alpha 1 antitrypsin in Liver Tissue*

issue	Frozen tissue		Paraffin embedded tissue			
	Without fixation	Prefixation for 18 months in buffered formalin	Fixation in buffered formalin for 4 h	24 h	7 days	18 months
		1 1600	1 12800	1 12800	1 12800	1 1600
		1 3200	1 25600	1 12800	1 12800	1 3200
	1 51200			1 25600	1 25600	

he table shows the highest dilution of anti alpha 1 antitrypsin which still gives a clear positive staining using indirect mmunoperoxidase staining technique

RESULTS

The influence of fixative fixation time and paraffin embedding was examined using IP technique on issue A–F

Results of investigations on tissue A–C are shown in Table 2 and 3 Table 2 shows that AAT was demonstrable in unfixed frozen sections using antisera in a lower concentration than in paraffin embedded formalin fixed sections No difference was observed between frozen sections of prefixed tissue and paraffin sections

The influence of the examined fixatives on the immunological reactivity of AAT is shown in Table 3 EA fixation at 4 °C caused the best preservation of AAT but the staining results were sometimes difficult to evaluate due to rather pronounced background staining – even when using extremely high dilutions of the primary antiserum Furthermore the tissue morphology was less satisfactory End point titres for 10% buffered formalin and AAF were lower than for EA while titres for Bouin s and Clarke s fixatives were clearly lower than for the afore mentioned In Table 3 it is furthermore seen that the fixation time did not have any essential influence on the preservation of the immunoreactivity when using fixation times be tween 4 hours and 7 days Extremely long fixation up to 18 months caused a clear decrease in the amount of demonstrable AAT (compare Table 2)

The effect of pretreatment of sections from for malin fixed paraffin embedded material with pro teolytic enzymes before the immunoperoxidase staining is shown in Table 4 Treatment with trypsin had no effect on tissue fixed up to 24 hours while treatment with protease increased the amount of demonstrable AAT Even more pronounced was the effect on tissue which had been fixed for extremely long time in formalin as it was in this case possible by using trypsin and especially using protease to unmask antigens in such a degree that the amount of demonstrable AAT was the same as demonstrated after 4 hour s fixation

The comp

ghtly more sensitive than the PAP technique In general more background staining was found when using PAP technique than with IP technique The

ivity of Alpha 1 ant trypsin in Liver Tissue

s fixative		Bouin s fixative			96% ethanol + 1% acetic acid (4 °C)		
h	7 days	4 h	24 h	7 days	4 h	24 h	24 days
00	1 800	1 3200	1 3200	1 3200	1 51200	1 51200	1 51200
00	1 3200	1 6400	1 3200	1 3200	1 102400	1 51200	1 51200

a clear positive staining using ind rect immunoperoxidase technique

TABLE 4 *Influence of Pretreatment with Proteolytic Enzymes on the Immunological Reactivity in Formalin Fixed Liver Tissue*

Fixation time	Without pretreatment	Pretreatment with trypsin	Pretreatment with protease
4 h	1 25600	1 25600	1 51200
24 h	1 12800	1 12800	1 51200
18 months	*1 3200*	*1 12800*	*1 51200*

The table shows the highest dilution of anti alpha 1 antitrypsin which still gives a clear positive staining using indirect immunoperoxidase staining

background staining was especially annoying in tissue fixed in EA IF technique showed in a few cases a slightly weaker staining than the two peroxidase techniques Autofluorescence did not cause trouble and the yellow autofluorescence from lipofuscin was *clearly discernible from* specific yellow green fluorescence in relation to AAT

Comparing the results obtained on tissue with and without globules *it was found* that a much higher concentration of anti AAT was needed for demonstration of AAT in tissue *without globules* than for AAT in tissue with globules

Comparing *the staining results obtained by using* IP technique on autopsy and biopsy material more AAT was found in autopsy material than in biopsy material both as more stained cells and as increased staining intensity of the individual *cell In other* respects the staining patterns were however concordant in the two materials

Control stainings by using absorbed anti AAT

TABLE 5 *Comparison of the Sensitivity of Different Immunohistochemical Techniques for Demonstration of Alpha 1 antitrypsin i Liver Tissue*

Immuno histochemical technique	Buffered formalin	Bouin s fixative	96 % ethanol + 1 % acetic acid (4 °C)
IP	1 25600	1 3200	1 51200
PAP	1 12800	1 1600	1 25600
IF	1 12800	1 800	1 25600

The table shows the highest dilution of anti alpha 1 antitrypsin which still gives a clear positive staining IP Indirect *immunoperoxidase technique* PAP Peroxidase antiperoxidase technique IF Indirect immunofluorescence technique

(control no 1) were negative in all biopsy materia and *in* autopsy material without globules Ir *autopsy material with globules positive reaction* could be demonstrated by using high concentration *of the absorbed antiserum* The reaction was always weaker by a factor ten than that obtained by using *non absorbed antiserum Using* immunoglobulin from non immunized animals and by replacing the primary antiserum with PBS negative staining was obtained in all cases

DISCUSSION

Following the first description of AAT deposits in liver tissue from patients with AAT deficiency of phenotype ZZ (24) this observation has been confirmed in a number of investigations using direct and indirect IF techniques on cryostat sections of fresh frozen or formalin fixed material (1 5 8)

Palmer (17) showed deposits of AAT in liv tissue from patients with phenotype MZ usi *direct immunofluorescence technique* on formal fixed paraffin embedded tissue Later on *performed a comparative investigation of stainin* on cryostat and paraffin sections of formalin fix tissue with and without globules using IP PAP ar IF techniques (18) AAT was demonstrated i cryostat sections of formalin fixed tissue wi globules using all three techniques Using IP ar PAP techniques positive AAT staining was found i formalin fixed paraffin embedded material wi globules but no staining with IF technique

Ray & Desmet (19) did not find any differenc between AAT staining in unfixed frozen section and sections of Bouin fixed paraffin embedde material using IF technique

None of the above mentioned investigators were able to demonstrate AAT in liver tissue withou globules In contrast *Feldman & al* (7) using IF technique on cryostat sections of paraformaldehyde fixed material demonstrated AAT deposits in liver tissue both with and without globules

A quantitative comparison of the influence of tissue preparation on the immunoreactivity of AAT does not seem to have been described previously In
... AT has been demonstra
... all
... ns

and on frozen and paraffin sections of formalin fixed tissue A slightly better preservation of the *immunoreactivity of AAT* was found in frozen sections of unfixed tissue than in paraffin sections of *formalin fixed tissue*

A comparative investigation of the influence of different fixatives on the preservation of the

mmunoreactivity of AAT has not previously been performed In this investigation a number of fixatives has been compared by using the same staining technique and the same tissue EA fixation at 4 °C and fixation in 10% phosphate buffered formalin or Lillie s AAF gave better results than the employment of Bouin s and Clarke s fixatives EA fixation however caused high background staining and less satisfactory tissue morphology

The fixation time had only a slight influence when using a fixation period less than a week while fixation for an extremely long period caused a clear reduction of demonstrable AAT Thus fixation for up to 24 hours in 10% phosphate buffered formalin is recommendable for the immunohistochemical demonstration of AAT in liver tissue as it combines preservation of AAT immunoreactivity and tissue morphology with low background staining in a satisfactory way

The employment of proteolytic enzymes for unmasking antigen determinants was originally introduced in connection with immunofluorescence staining of formalin fixed paraffin embedded tissue (10) The enzymes have recently also been used in connection with immunoperoxidase staining In accordance with previous investigations (10 11) I found that pretreatment with trypsin and protease especially of tissue which have been fixed for extremely long time is able to increase the amount of demonstrable AAT in paraffin sections Unmasking with proteolytic enzymes may be used with advantage in cases where only small amounts of AAT are accumulated in the liver tissue as e g in biopsies from heterozygous MZ patients without PAS/diastase resistant globules

Opinions have previously differed as regards the suitability of the three different immunohistochemical techniques for demonstration of AAT in liver tissue *Palmer* (18) found that the two immunoperoxidase staining techniques were better than IF technique and that PAP technique was better than IP technique *Triger & al* (25) likewise found that immunoperoxidase technique was more suitable than immunofluorescence technique when using paraffin sections In contrast *Blenkinsopp* (3) found that IP technique was inferior to both IF technique and PAP technique

In this investigation the comparison of IP PAP and IF technique has been performed using the same primary antiserum and the same tissue and in this case no convincing difference between the techniques has been demonstrated The sensitivity of IP PAP and IF techniques was for practical purposes equal

The different opinions as regards sensitivity and suitability of the three techniques are probably due to difference in quality of the used immunological reagents as for example differences in conjugation degree and antibody titre

In conclusion the employment of IP staining on formalin fixed paraffin embedded material for demonstration of AAT in liver tissue has been shown to give satisfactory results as it combines a sensitive staining technique with good preservation of immunoreactivity and morphology of the tissue

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PRECISION OF HISTOLOGICAL GRADING OF MALIGNANCY

Sources of Variation in a Histological Scoring System for Grading Cancer of the Larynx

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Græm N Helweg Larsen K. & Keiding N Precision of histological grading of malignancy Sources of variation in a histological scoring system for grading cancer of the larynx Acta path microbiol scand Sect A *88 307–317 1980*

The precision of a grading system based upon average score of 8 items for histological evaluation of malignancy of epidermoid carcinomas of the larynx was studied by letting 6 pathologists examine biopsies from 22 new cases Exploratory statistical tools as well as a statistical framework (variance component models) for separating and evaluating the noise components are suggested The order of magnitude of the resulting overall measurement uncertainty allowed discrimination between *patients* though if a patient is graded by only *one* pathologist only results at the extremes of the scale of malignancy should be trusted as possible therapeutical guidance Based upon an analysis *of the basic structure* of the grading system the single noise components making up the measurement uncertainty were determined Of greatest importance was noise created by disagreement between the pathologists in their ranking of the patients on the scale of *malignancy Furthermore the* pathologists used the individual items differently and certain items seemed to conform little to the scoring system An attempt to minimize these two last components was *performed however* neither calibration of the pathologists nor omission of the deviating items improved the precision significantly

Key words Cancer of the larynx variance component models histological grading

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Histopathological grading has the double purpose of refining and objectifying histopathological diagnosis Like almost any diagnostic statement, the result of a histopathological grading is only partially correlated to prognosis but in contrast to laboratory measurements which are often quite close to ideal objectivity (observer independent) histopathological grading is in addition disturbed by the subjective nature of the procedure being based upon a subjective interpretation of a *complicated image* This subjective factor may be [illegible] sumed to be more critical when the refined statements of histopathological *grading* are aimed at than for the less subtle traditional histopathological diagnoses

The important role of the observer in histopathological grading as compared to e g laboratory measurements necessitates more detailed definition and evaluation of the precision and accuracy of the method Of course the final test of any diagnostic procedure is how it correlates to prognosis In this report, however, we define and *evaluate the intrinsic* precision of histopathological grading in the particular context of epidermoid carcinoma of the larynx

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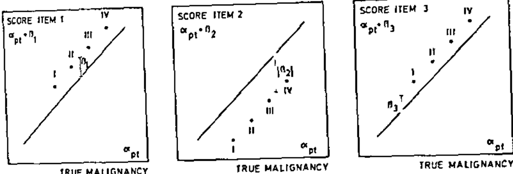


Fig 1 Theoretical score patterns for a perfect scoring system (three items four patients = I IV one pathologist $\beta_1 + \beta_2 + \beta_3 = 0$)

and agreed on an interpretation of the individual scores as described by *Helweg Larsen et al* (1978)

Statistical Analysis

The statistical evaluation will consist of 2 parts First an exploratory data analysis investigating the suitability of each of the 8 items the varying use of the system by the 6 pathologists and the consistency of the grading in relation to a one dimensional concept of histological malignancy thus forming a natural basis for the use of a simple average of scores as measure of malignancy Secondly a definition and assessment of the precision of the grading system should one want to use it routinely Finally on the basis of these results an attempt to point out some ways to improve the grading system

RESULTS

Exploratory Analysis of the Structure of the Grading System

The perfect grading system It is natural to base the use of the average score as an indicator of malignancy upon an assumption of one-dimensional histological malignancy changes in malignancy level should affect all items in the same way though it is no requirement that for instance 2 on one item corresponds to 2 on another Were the true malignancies of a series of patients known we should therefore expect a pattern like that of Fig 1 which indicates how in a perfect grading system the scores on each individual item increase with increasing true malignancy Further if an unweighted average is to be used as malignancy index each item should discriminate equally well between slight and severe cases This property is indicated by the parallelity of the three lines that could be drawn through the 4 points in each diagram In addition to this the reproducibility of the scores should be perfect

Deviations of the present scoring system from the idealized pattern In the actual data the semiquantitative nature of the scoring system with only 4 response categories makes diagrams like those of Fig 1 unfeasible for each pathologist and item We shall therefore first compare items pooled over pathologists and secondly pathologists pooled over items Since the true malignancy is of course an abstraction we use instead the average score over items and pathologists as reference (abscissa) Fig 2 shows for each item the average score over the 6 [illegible]

responses missing with a distribution on the items 1–8 of respectively 1 2 1 3 2 14 10 and 2 In the following analysis we treat those as ordinary missing values and substitute expected values by a routine procedure The adequacy of that assumption in the context is discussed further below)

It is seen from Fig 2 that items 1 3 5 and 7 follow the general pattern determined by the average quite closely It also appears that the score on item 7 is consistently below the full drawn line as is indeed permitted cf Fig 1 Items 2 and 8 follow the general pattern but much less accurately Finally items 4 and 6 are generally scored rather independently of the remaining ones

In a similar fashion we plot in Fig 3 the average over items of each pathologist s scores for a given patient again using the overall patient average as reference First it is seen that the pathologists agree well in their overall level (most points being close to the identity line) except pathologist F who generally has higher scores than the average opinion Closer inspection further reveals differences between the pathologists in their ability or willingness to discriminate between the patients Thus pathologists A and B keep close to the average opinion whereas C and F discriminate more and D and E less than the average

MATERIAL AND METHODS

Biopsies and Histological Grading

The basis of this study is the biopsies from 22 consecutive patients with a new diagnosis of epidermoid carcinoma of the larynx primarily seen at the Department of Otology Kommunehospitalet Copenhagen during 1977 The biopsies were fixed in formalin and cut in 12 5μm sections of which 9 were stained by haematoxylin eosin and 3 by van Gieson alcian

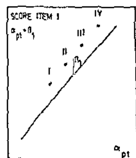
Immediately after the routine diagnostic procedures the biopsies were graded once by each of 6 pathologists below designated A–F working blindly and independently The grading was performed according to a histological scoring *system for evaluation of malignancy* of epidermoid carcinoma of the larynx The system was set up by *Jakobsson* (1973 a and b) and later modified by *Lund et al* (1976) and is based upon 8 items or questions (*Table 1*) *Items 1 2 3* and *4 vi* Structure Differentiation Nuclear polymorphism and Mitoses deal with the tumour cells alone whereas 5 6 7 and 8 describing Mode and Stage of invasion Vascular invasion and Cellular response treat the relations between the tumour cells and the host tissue

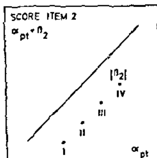
Each of the 8 items has 4 alternative answers which are scored 1 2 3 or 4 *If the actual histological material* is unsuitable for answering a certain item the item is omitted The average of the scores consequently ranging between 1 00 and 4 00 indicates the malignancy of the tumour

In order to achieve uniformity in the grading the pathologists discussed the system before the investigatic

Table 1 *Histological Grading of Malignancy*

Item number	*Tumour cell population*	Points			
		1	2	3	4
1	Structure	Papillary and solid	Strands	Small cords and groups of cells	Marked cellular dissociation
2	Differentiation	Highly keratinization	Moderately some keratinization	Poorly Minimum keratinization	Poorly no keratinization
3	Nuclear polymorphism	Few enlarged nuclei	Moderate number of enlarged nuclei	Numerous irregular enlarged nuclei	*Anaplastic* immature enlarged nuclei
4	Mitoses	Single	Moderate number	Great number	Numerous
	Tumour host relationship	Points			
		1	2	3	4
5	Mode of invasion	Well defined borderline	Cords less marked borderline	Groups of cells no distinct borderline	*Diffuse* growth
6	Stage of invasion	Possibly	Microcarcinoma (few cords)	Nodular into connective tissue	Massive
7	Vascular invasion	None	Possibly	Few	Numerous
8	Cellular response (plasmolymphocytic)	Marked	Moderate	Slight	None





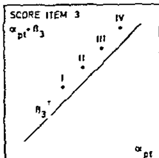


Fig 1 Theoretical score patterns for a perfect scoring system (three items four patients I–IV one pathologist $\beta_1 + \beta_2 + \beta_3 = 0$)

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Statistical Analysis

The statistical evaluation will consist of 2 parts First an exploratory data analysis investigating the suitability of each of the 8 items the varying use of the system by the 6 pathologists and the consistency of the grading in relation to a one-dimensional concept of histological malignancy this forming a natural basis for the use of a simple average of scores as measure of malignancy Secondly a definition and assessment of the precision of the grading system should one want to use it routinely Finally on the basis of these results an attempt to point out some ways to improve the grading system

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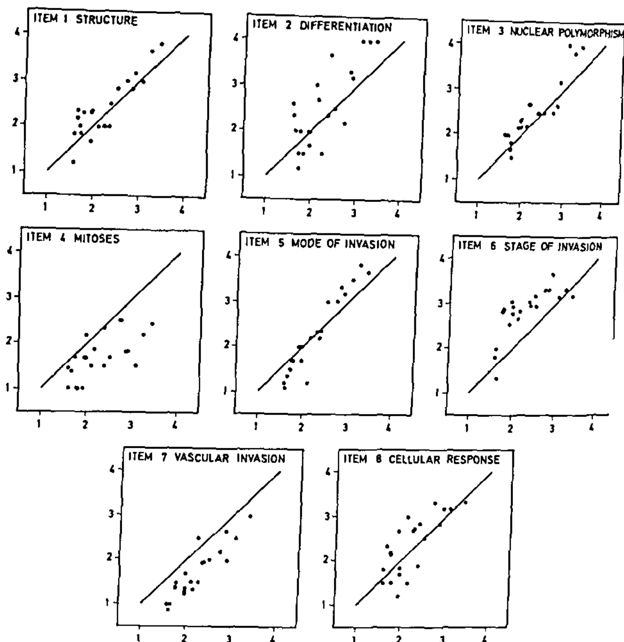


Fig 2 Plots of average score over pathologists for each item (ordinate) against the patient's estimated true malignancy (abscissa), given as the average score over pathologists and items (Each point corresponds to one patient)

Finally we use a similar approach to compare the usage of the items among the pathologists Fig 4 shows for each pathologist the average score over patients for a given item (indicated by its number) plotted against the average over pathologists and patients It is seen that the pathologists do not quite agree on the use of the individual items The extreme parts of the scale are used more than average by pathologists A and B and to some extent by E, and less than average by C Pathologists D and F seem to use the items differently (larger spread around a straight line) The pathologists in particular disagree on the use of items 4 and 7

Definition and Assessment of the Precision of the Grading System

We saw in the exploratory part of the analysis above that there were certain variations between the extent to which the various items and the various pathologists conformed to the general pattern In this section we want to quantify these variations in order to obtain a synthetic measure of the precision of the grading system

We take the viewpoint that the grading system is given and it is desired to estimate the malignancy level of a new patient The grading system is to be used by a pathologist belonging to a certain

Fig 3 Plots of average score over items for each pathologist (ordinate) against the patient s estimated true malignancy (abscissa)

Fig 4 Plots of average score over patients (ordinate) against the general item average (abscissa) One plot per pathologist each point corresponds to an item (indicated by its number)

population of »competent pathologists« from which the 6 present pathologists may be regarded a random sample This means that if pathologists do not use the items quite the same way, the *pathologist-item interaction* is an unavoidable »noise« in the system Similarly, minor disagreement between competent pathologists on the ranking of patients *(patient-pathologist interaction)* as well as *patient-item* interaction are unavoidable noise sources

The statistical model used is a three-way analysis of variance with certain random effects The present account is based on *Jensen* (1979) somewhat in line with *Nelder* (1977) A standard textbook treatment is by *Scheffe* (1959) We use variance component models primarily because no other available statistical tool can catch and synthesize the various sources of variations inherent in the problem Although the exposition is phrased in terms of the normal distribution, the emphasis is on identifying and calculating the separate variance terms, which does not depend critically on special distributional assumptions

The statistical model may be described by the following decomposition of the basic observation which is the score $X_{pt\,path\,i}$ (= *1, 2, 3 or 4*) of the pathologist path on item i, when grading the biopsy of patient pt

$$X_{pt\,path\,i} = \alpha_{pt} + \beta_i + U_{path} + V_{pt\,i} + Y_{pt\,path} + Z_{path\,i} + W_{pt\,path\,i}$$

where α_{pt} *and* β_i *are fixed numbers whereas the* U s [V s Y s,] are independent normally distributed random variables with zero expectation and constant variance ω_{path}[$\omega_{pt\,i}$ $\omega_{pt\,path}$]

The interpretation of the model is as follows We agree that a patient pt has a (latent) *true malignancy* α_{pt} and that the *ideal score* $\alpha_{pt} + \beta_i$ on item i for patient pt is obtained from the true malignancy by an *additive correction* β_i (cf Fig 1) (We thus adopt the convention that $\Sigma\,\beta_i = 0$) Deviations from this ideal additivity, the *patient item interaction* are here considered as (random) noise and hence modelled by a random variable $V_{pt\,i}$ whose variance measures the magnitude of the noise

The general deviation of a pathologist from the true score is also modelled as a random variable U_{path} This is because we want *the analysis to* be applicable to a *population of pathologists* and not only to the 6 specific participants in this study Similarly, the specific deviations $Y_{pt\,path}$ from true score of pathologist path when studying patient pt as well as the specific deviations $Z_{path\,i}$ from true *score* when pathologist path uses item i are modelled as random noise terms *Finally, even if all* the so far mentioned corrections were accounted for, there would still be the *possibility of a second* order interaction $W_{pt\,path\,i}$ which under the present set up also contains the basic measurement uncertainty of the instrument and thus provides a baseline against which to assess the significance of the other noise terms The estimation of the mean value parameters α_{pt} and β_i *(the systematic components)* as well as the variance parameters ω_A *(the random components)* is obtained fairly directly from a standard analysis of variance Table 2 shows the ordinary three way (systematic) analysis of variance table as well as the estimates of the patient means α_{pt} and item means $\bar{\alpha} + \beta_i$, $\bar{\alpha} = \Sigma\alpha_{pt}/22$ (As mentioned above, missing values are filled in by standard prediction methods in the three way anova model In the »degrees of freedom« column it is *indicated in brackets how many degrees of freedom* have been sacrificed in each variance component due to missing values)

The »mean square« column in the anova table contains estimates of parameters σ_A^2 which are connected to the parameters ω_A defined above by the relations (cf *Jensen* 1979)

$$\omega_{pt\,path\,i} = \sigma^2_{pt\,path\,i}$$

$$\omega_{pt\,path} = \frac{1}{n_i}\left(\sigma^2_{pt\,path} - \sigma^2_{pt\,path\,i}\right)$$

where n_i = *number of items* = *8* and similarly for $\omega_{pt\,i}$ and $\omega_{path\,i}$ and finally

$$\omega_{path} = \frac{1}{n_i n_{pt}}\left(\sigma^2_{path} - \sigma^2_{pt\,path} - \sigma^2_{path\,i} + \sigma^2_{pt\,path\,i}\right)$$

In this way the parameters ω_A may be estimated as mentioned in the discussion a negative estimate may well be interpreted in an *alternative statistical* framework The results are shown in Table 3

We are now ready to compute the measurement uncertainty of the grading system The patient true malignancy α_{pt} is to be estimated by the patient average $\bar{X}_{pt}$ We use the decomposition above to calculate the variance (summing the *individual* independent components) as follows Using the assumption $\sum_i \beta_i = 0$ we have

$$\bar{X}_{pt} \sim \alpha_{pt} + \bar{U} + \bar{V}_{pt} + \bar{Z} + \bar{W}_{pt}$$

where a bar as usual denotes average thus

$$\bar{V}_{pt} - \frac{1}{n_i}\sum_i V_{pt\,i}, \quad \bar{Z} - \frac{1}{n_{path}\,n_i}\sum_{path}\sum_i Z_{path\,i}$$

Hence

$$\mathrm{Var}(\bar{X}_{pt}) = \frac{\omega_{path}}{n_{path}} + \frac{\omega_{pt\,i}}{n_i} + \frac{\omega_{pt\,path}}{n_{path}} + \frac{\omega_{path\,i}}{n_{path}\,n_i} + \frac{\omega_{pt\,path\,i}}{n_{path}\,n_i}$$

$= 0\ 0304$

corresponding to a standard error of 0 174

TABLE 2 *Three Way Analysis of Variance*

Source of variation	Degrees of freedom (missing values)	Sum of squares	Mean square	F (against residual)	P
Patients	21	309 59	14 742	54 4	< 0001
Pathologists	5	4 47	0 893	3 30	006
Pt. × path	104 (1)	71 65	0 689	2 54	< 0001
Items	7	148 03	21 147	78 1	< 0001
Pt. × items	147	151 47	1 030	3 80	< 0001
Path × items	35	59 64	1 704	6 29	< 0001
Residual	701 (34)	189 89	0 271		
Total	1020	934 74	0 916		

Patient average scores (ranked)

1 60	1 61	1 69	1 75	1 76	1 79	1 93	1 98	1 99	1 99
2 12	2 17	2 27	2 39	2 41	2 52	2 75	2 85	2 92	3 10
3 25	3 43								

Item average scores

Item	1	2	3	4	5	6	7	8
Item av score	2 41	2 46	2 51	1 68	2 26	2 85	1 71	2 40

If data from only one pathologist are used (but we still want to include our knowledge regarding the pathologist population variation) we use $\bar{X}_{pt\ path}$ as estimate of α_{pt} and

$$\mathrm{Var}(\bar{X}_{pt\ path})$$
$$= \omega_{path} + \omega_{pt\ path} + \frac{\omega_{pt} + \omega_{path} + \omega_{pt\ path}}{n} = 0\ 103$$

s e − 0 321

Table 3 *Estimated Variance Components*

	d f	σ^2	ω
path	5	0 894	−0 007
pt i	147	1 030	0 127
pt path	104	0 689	0 052
path i	35	1 704	0 065
pt path i	701	0 271	0 271

Attempts to Improve the Precision of the Grading System

Consequences of omitting some items In the exploratory analysis we saw that items 4 and 6 and to a lesser extent items 2 and 8 did not seem to contribute relevantly to the malignancy dimension here considered In order to assess the consequences of omitting these items we re run the analysis of variance assuming that the grading system has only

Table 4 *Estimated Variance Components when only Items 1 3 5 and 7 Are Retained*

	d f	σ^2	ω
path	5	3 114	0 016
pt i	63	0 506	0 042
pt path	104	0 646	0 097
path i	15	1 281	0 046
pt path i	302	0 257	0 257

population of »competent pathologists« from which the 6 present pathologists may be regarded a random sample This means that if pathologists do not use the items quite the same way, the *pathologist-item interaction* is an unavoidable »noise« in the system Similarly, minor disagreement between competent pathologists on the ranking of patients *(patient-pathologist interaction)* as well as *patient-item* interaction are unavoidable noise sources

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$$\bar{X}_{pt} = \alpha_{pt} + \bar{U} + \bar{V}_{pt} + \bar{Z} + \bar{W}_{pt}$$

where a bar as usual denotes average thus

$$\bar{V}_{pt} = \frac{1}{n_i}\sum_i V_{pt\,i} \qquad \bar{Z} = \frac{1}{n_{path} n_i}\sum_{path}\sum_i Z_{path\,i}$$

Hence

$$Var(\bar{X}_{pt}) = \frac{\omega_{path}}{n_{path}} + \frac{\omega_{pt\,i}}{n_i} + \frac{\omega_{pt\,path}}{n_{path}} + \frac{\omega_{path\,i}}{n_{path} n_i} + \frac{\omega_{pt\,path\,i}}{n_{path} n_i} = 0\ 0304$$

corresponding to a standard error of 0 174

< 2 5 - 1 6 4 × 0 3 2 - 1 9 8 should we recommend against 1 64 being the 95 fractile in the standard normal distribution In the present series of patients this would mean that 11/22 = 50% would be »safely« allocated If 6 pathologists performed the grading the interval would be narrowed to 2 21 – 2 79 (2 50 ± 1 64 × 0 174) so that 17/22 = 77% of the patients were allocated with safety in accordance with the intention

These examples have important implications Firstly an evident consequence would be that it is desirable to have several pathologists to grade each patient (In addition this procedure would be a precaution against a careless extrapolation of the inferences about a population of competent patho logists drawn from the present sample« of 6) The second implication is that only the extremes of the scale should be trusted

The Sources of Variation

The analysis of the basic structure of the scoring system and the subsequent analysis of the variance components have been utilized to localize and quan tify the sources of noise in the grading system Be fore the discussion of the individual sources it is in structive to consider the decomposition of the basic measurement uncertainty probably best expressed as the variance if one pathologist grades one patient The estimated malignancy (average score) has a variance given by

$$V(\bar{X}_{pt\,pa\,h})$$

$$\omega_{pa\,h} + \omega_{pt\,pa\,h} + \frac{\omega_{p} \quad + \omega_{pa\,h} \quad + \omega_{p\quad pa\,h}}{n}$$

$$- 007 + 052 + \frac{127 + 065 + 271}{8}$$

$$- 007 + 052 + 016 + 008 + 034$$

$$103$$

It follows that the main sources of variation are the general variance (noise) term $\omega_{p\quad pa\,h}/n$ 271/8 034 and the patient pathologist inter action $\omega_{pt\,pa\,h}$ 052 The interactions with items are less important because the estimated malignancy is computed by averaging over 8 items but not averag ng over pathologists

D vergence among the Pathologists in Their Evaluat on of the Patients

Slight deviation from the general level and d fference in the d scrimination between the patients were seen (Fig 3 and Table 3) but it appeared from the example above that the pathologist patient inter action has a relatively great weight in the final computation of the uncertainty of the grading system This divergence may reflect how the individual pathologist acts in case of doubt which easily may arise as the four scoring categories of each item do not represent well-defined separate entities but rather indistinct parts of a continuum

Deviation from the general opinion may as suggested by *Iversen & Sandnes* (1971) reflect the therapeutical philosophy of the pathologist so that a therapeutical optimist instinctively overestimates malignancy and vice versa Perhaps this effect would become more pronounced if it was known by the pathologists that the grading would have important therapeutical implications unlike the conditions of the present investigation

Deviations from One dimensionality of Histological Malignancy

In the following considerations it is important to realize the *relative* role of estimated true malignancy in this study Our intrinsic comparison has no choice but to use the average score as representing »truth« As an extreme case suppose that in fact only item 1 really represented malignancy while the other seven while highly correlated represented varying degrees of something else Then any internal comparison would imply that item 1 ought to be omitted

It was seen from Fig 2 that items 2 4 6 and 8 affect the one-dimensional concept of histological malignancy measured by the present grading sys tem This becomes even more convincing when it is taken into account that we used the average of all 8 items (including the one under study) as abscissa From Table 2 it appears that the item average scores differ significantly (range - 1 68 – 2 85) This is a question of score category definition which does not detract from the consistency of the system however it should be noted that these differences are ignored when an item which cannot be evaluated and therefore is omitted in the calculation is ascribed a value equal to the average of the remaining items This inconsistency has been [illegible]

[illegible] to be made in this connection First this procedure cannot reasonab[illegible]

[illegible] not in itself provide information an assumption which is perhaps not completely satis fied here Since only 35 out of 1056 responses were missing here we expect any bias to be rather minor but in series of less suitable biopsies noise may still arise in this way

TABLE 5 *Estimated Variance Components when Pathologist Main Effects and Pathologist-Item Interactions Are Considered Non-Random*

	d f	σ^2	ω
pt i	147	1 030	0 127
pt path	104	0 689	0 052
pt, path i	701	0 271	0 271

4 items namely 1, 3, 5 and 7 The results are presented in Table 4 As above we then compute

$$\mathrm{Var}(\bar{X}_{pt}) = 0\,0418, \text{s e} \approx 0\,205,$$
$$\mathrm{Var}(\bar{X}_{pt\,path}) = 0\,200, \text{ s e} = 0\,447$$

Calibration of pathologists We consider an idealized case of adjustment, that is, the pathologist main effect (formerly U_{path}) and the pathologist-item interaction (formerly $Z_{path\,i}$) now designated γ_{path} resp $\delta_{path\,i}$ can be estimated without error

The model may then be specified as

$$X_{pt\,path\,i} = \alpha_{pt} + \beta_i + \gamma_{path} + \delta_{path\,i} + V_{pt\,i} + Y_{pt\,path} + W_{pt\,path\,i}$$

From the analysis of variance in Table 2 we may now derive the estimated variance components in Table 5 We then get

$$\mathrm{Var}(\bar{X}_{pt}) = \frac{\omega_{pt\,i}}{n_i} + \frac{\omega_{pt\,path}}{n_{path}} + \frac{\omega_{pt\,path\,i}}{n_{path}\,n_i} = 0\,0302$$

s e 0 174,

$$\mathrm{Var}(\bar{X}_{pt\,path}) = \omega_{pt\,path} + \frac{\omega_{pt\,i} + \omega_{pt\,path\,i}}{n_i} = 0\,102$$

s e 0 319

DISCUSSION

Before entering into the details of the results of this investigation let us repeat from the introduction that our only aim here is to study the intrinsic consistency and precision of the grading system Since we do not know any way of directly observing »true malignancy« the only possible external comparison would be to the observed prognosis For this to be informative a different study containing many more patients and a considerable follow-up period would be necessary

Statistical Model

There are several alternative approaches to the definition of analysis of variance models with random effects Although the definition in terms of specifying the complete covariance structure (such as »intraclass correlation«) may be the more satisfactory from a statistical point of view, we have here used an error component formulation in the hope that it is more easily accessible for the readers of the present paper It should however be borne in mind that »negative variances« of all other error components than the residual ($W_{pt\,path\,i}$) may well be interpreted in the former framework

In the presentation of the variance component model above, we performed no significance tests to obtain a possible simplified model First, it is immediately seen from Table 2 that all main effects and first-order interactions are highly significant against the residual given by the second order interaction We draw one important inference from this fact Any »rounding error effect« of the integer nature of the basic score would increase the residual variance Since this is still by far the smallest, that objection against using the normal distribution can reasonably be disregarded Secondly the question of the negative estimate $\omega_{path} = -\,007$ (first model) may be treated by calculating an approximate standard error ω_A being a linear combination of χ^2 – statistics This leads to $\omega_{path} = -\,007 \pm 004$ meaning that we may well assume $\omega_{path} = 0$ and thus have no need for a further discussion of the alternative statistical framework

Basic Measurement Uncertainty

It was seen that if data from only one pathologists were used the histological grading was perturbed by a standard error of $\sqrt{0\,103} = 0\,321$ If 6 pathologists were involved the standard error became 0 174

Any use of the grading system as therapeutical guidance should consider the relatively good prognosis of epidermoid carcinoma of the larynx treated by radiation alone (*Sand Hansen* 1975 *Jørgensen* 1976) as well as the severity of any possible surgical intervention *Lund et al* (1977) suggested that »In cases where exploratory operations on the regional lymph nodes are considered the histological score may be the decisive factor« To illustrate the implications of the measurement uncertainty of the grading system on its use as an aid in such decisions let us assume that it is established that patients with true score greater than 2 5 should be operated while patients with true score less than 2 5 should not Assume further that we want to make no more than 5 % misclassifications in the long run and that only one pathologist performs the grading Then if the patient's observed average score was $> 2\,5 + 1\,64 \times 0\,32 = 3\,02$ we should recommend operation and only if it was

one pathologist grades each patient One recommendation drawn from the study is that the observer patient interaction seems so large that each biopsy should be graded by several pathologists

From a practical point of view the total variability of a grading system like this is composed of the uncertainty of prognostic statements for given levels of malignancy on the one hand and the intrinsic lack of precision on the other The present paper has only studied the latter source of variation

We are grateful for several useful discussions on variance component models with *Søren Tolver Jensen* Institute of Mathematical Statistics University of Copenhagen and we thank *Poul T Bøesen Claus Petri Carl O Povlsen* and *Jørgen Rygaard* for their grading of the biopsies and for the inspiring interest they took in the investigation *Jørgen Hilden* supplied useful comments on the manuscript

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It might be that the items Keratinization Mitoses, Stage of invasion and Cellular response correlate well to clinical malignancy as reported by *Jakobsson* (1973), but in the grading system they are important sources of noise and main contributors to the patient-item interaction ω_{pi} (Table 2 vs 4) In order to increase the precision of the grading we omitted these items (disregarding a possible psychological interactive effect between retained and omitted items), but found that the grading thereby became less precise The patient-item interaction certainly was reduced to one third (Table 4) of the original value, but at the expense of a doubling of the patient pathologist interaction which in the present example has 4 times (number of items) greater numerical weight in the final calculation of the standard errors (cf example above)

Presumably an imperfect one-dimensionality, which is not surprising remembering the diversity of malignant tumours, also blurs the results of other histological grading systems This may be expected not only in those employing a single sum or average of scores as the system for grading of breast carcinomas of *Bloom & Richardson* (1957), but also in grading systems in which the »computation« is performed »within the pathologist« by a complex psychological process e g in grading of atypia of bladder tumours (*Bergkvist et al* 1965) and dysplasias of the uterine cervix (*Poulsen et al* 1975)

In order to get an optimal consistency in a grading system analytical diagrams like those of Fig 2 would form a sound basis of the design Of course it should be remembered that other arithmetical operations than simple addition of score values are possible and that the score values may be weighted differently

Divergence among the Pathologists in Their Scoring of the Individual Items

It was seen from Fig 4 and Table 3 that the pathologists did not agree in their use of the individual items especially pathologist D and F dissented from the average, and items 4 (Mitoses) and 7 (Vascular invasion) were scored with great disagreement The divergence on mitotic activity is remarkable as this theoretically might be easily quantitated and as this parameter is often used in grading of malignancy As pointed out by *Silverberg* (1976) it might be due to a disagreement on the morphological definition of mitoses The disagreement on item 7 may have a similar cause The definition of especially score 2 on this item is vague While the term »possibly« on item 6 (Stage of invasion) is fairly well understood it has a rather diffuse meaning in case of vascular invasion as the biopsies contained few and small vessels Ten out of 35 missing values occurred in item 7 Perhaps it would have been reasonable to drop this item in still more cases, but just the escape option »possibly« may have prevented this These examples show that the consistency of diagnostic parameters should be secured before they are introduced in routine pathology

In another attempt to find ways of increasing the precision of the grading system we imagined the pathologists to be calibrated In principle – given access to a large sample of scorings – one might for each pathologist determine his/her particularities (in the analysis expressed as γ_{path} and $\delta_{path\,i}$) to a standard form However it was noticed that even perfect adjustment of pathologists would have very limited effect as the basic measurement uncertainty expressed as the standard deviation of $\overline{X}_{pi\,path}$ was only reduced from 0 321 to 0 319

Variance due to Preconception

It would be interesting to have further psychological information on how the pathologists make up their minds about a given biopsy – proceeding from the items to a conclusion (such as implicitly assumed when using the grading system) or by first forming a general opinion about »how malignant is this tumour?« and subsequently filling in the score values in accordance with the conclusion Obviously, we do not have such psychological insight It might however be of interest to point out that the parameter $\omega_{pi\,path}$ may be interpreted as the »pure« covariance between the responses on two different items by a given pathologist on a given patient that is for given general levels of the patient, the pathologist and the items and given interactions between patient and items and between pathologist and items

The highly significant positive intrinsic covariance $\omega_{pi\,path}$ between responses on different items on a given patient by a given pathologist may be interpreted as an indication that the pathologists do not score the items independently but at least in some cases proceed *from* a general impression *to* the individual item

CONCLUSION

This paper has defined and discussed methodology for studying consistency and precision of histopathological grading systems taking particularly into account the important observer component in the measurement uncertainty In the particular example studied, a grading system concerning epidermoid carcinoma of the larynx was seen to be able to discriminate between extreme cases, even if only

SURVIVAL IN IDIOPATHIC GLOMERULONEPHRITIS

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Nyberg M Pettersson E Tallqvist G & Pasternack A Survival in idiopathic glomerulonephritis Acta path microbiol scand Sect A 88 319-325 1980

Actuarial survival was studied in 285 adult patients with idiopathic glomerulonephritis (GN) Minimum follow up was 7 years 105 patients had minimal change GN (MC) 22 membranous GN (MGN) 20 acute GN (AGN) 11 mesangial sclerosis GN (MSGN) 28 mesangiocapillary GN (MCGN) 8 crescentic GN (RPGN) 27 unclassifiable GN 61 focal proliferative GN (FGN) and 3 focal segmental glomerular sclerosis and hyalinosis (FSGSH) Ten year survival was best in FGN (91%) and progressively poorer in MC (90%) AGN (85%) MSGN (83%) MGN (82%) MCGN (62%) unclassifiable GN (37%) and RPGN (16%) One of the three patients with FSGSH died during follow up At 10 years survival differed significantly ($p < 0.01$) from expected only in MCGN RPGN and unclassifiable GN Our results suggest that – because survival was not significantly different from expected in most types of GN – the current classification of GN is only a crude guide to prognosis The nephrotic syndrome was found to worsen prognosis in MGN and MCGN

Key words Glomerulonephritis nephrotic syndrome survival renal biopsy

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Although many clinicopathologic correlation studies of histopathologically-defined glomerulonephritis (GN) have included data on survival only in more recent investigations (*Hardwicke & al* 1967 *Antoine & Faye* 1972 *Cameron & al* 1973 *Habib & al* 1973 *Habib & al* 1975 *Row & al* 1975 [illegible] survival (*Cutler & Ederer* 1958 *Anderson & al* 1974) been used that render results from different studies comparable These investigations however have dealt with only one or a few histopathologic types of GN Survival of excluded or unclassifiable patients has not been accounted for To obtain a more complete picture of mortality from idiopathic GN we believe that survival should be analysed in a comprehensive series that encompasses all histopathologic types including unclassifiable cases There is only one report of this kind (*Cameron* 1972) but it does not describe the age distribution of the patients A study of children has been published (*Habib* 1970) for which actuarial survival was analysed later (*Cameron* 1973) together with data on survival from other clinicopathologic studies by other authors We have found no study of this kind describing survival in exclusively adult GN patients Prognosis has been considered to be predictable from the histopathologic type of GN (*Habib* 1970 *Habib* 1975, *Cameron* 1972) However there are conflicting reports on survival in some types of GN (*Cameron* 1973) especially in membranous GN (*Cameron* 1972 *Cameron* 1973, *Legrain & al* 1978 *Noel & al* 1979)

The purpose of this study was to evaluate the relation of the currently used light microscopic classification of GN to long term survival in a comprehensive adult patient population Survival results were also compared with expected survival in a matched normal population

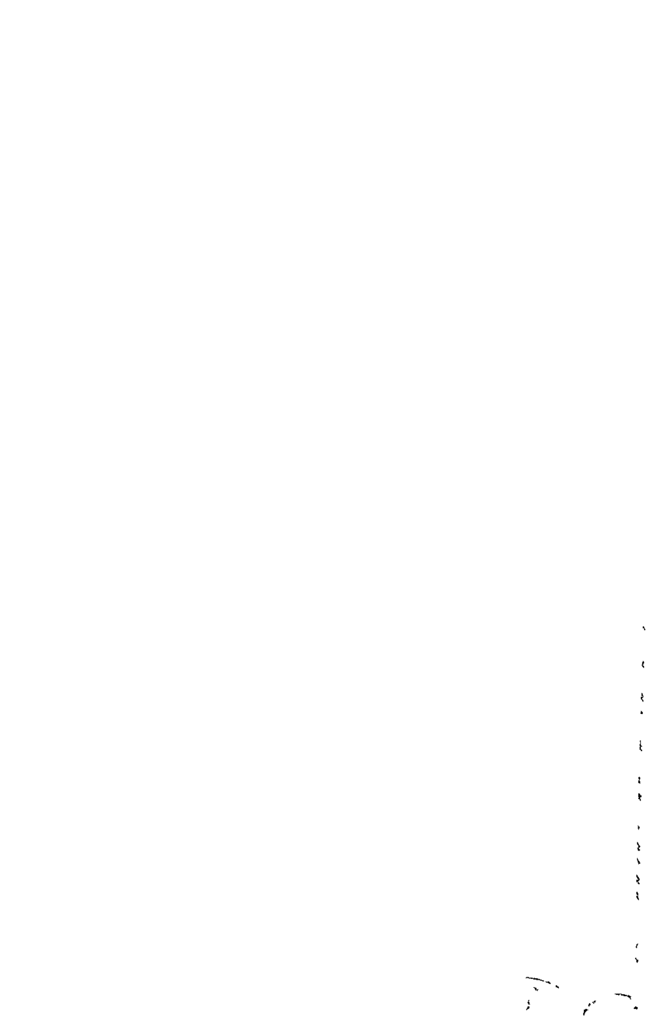


TABLE 1 *Clinical Data*

	Number of patients	Males/females*	Age (years)			Signs*						Steroid treatment*	Lost to follow up*
			mean	median	range	proteinuria	haematuria	proteinuria & haematuria	nephrotic syndrome	renal insufficiency or renal failure	hypertension		
Minimal change	105	48/57	32	29	16–66	31	22	31	19	2	–	19	–
Membranous GN	22	15/7	38	32	16–68	9	–	1	11	3	7	12	–
Acute GN	20	15/5	46	49	26–71	1	4	9	7	8	6	11	–
Mesangial sclerosis	11	5/6	37	31	16–61	1	3	5	–	2	4	2	1
Mesangiocapillary GN	28	16/12	35	33	15–70	8	5	7	10	7	12	10	1
Crescentic GN	8	6/2	51	52	38–73	–	2	5	2	5	4	4	1
Unclassifiable GN	27	17/10	44	41	17–76	9	12	11	4	9	7	7	1
Focal proliferative GN	61	30/31	39	39	16–71	14	7	22	1	6	14	11	3
Focal segmental glomerular sclerosis & hyalinosis	3	2/1	35	28	27–50	1	1	–	–	–	1	–	–
Total series	285	154/131	37	34	15–73	74	56	91	54	42	55	76	7

* Number of patients

MATERIAL AND METHODS

Selection of patients From all percutaneous renal biopsies referred to the Department of Pathology, Maria Hospital, during the period 1959 through 1971, we selected those that were reported, on light microscopy, to be normal or to show changes compatible with or suggestive of GN These biopsies were then re-examined microscopically without prior knowledge of clinical or histological data All biopsies were excluded that originally or at re-examination had less than five glomeruli (unrepresentative) Excluded were also all biopsies in which 50 per cent or more of the glomeruli were hyalinised (end stage lesions of uncertain glomerular or non-glomerular origin) The biopsies unrepresentative at re-examination were randomly distributed among histopathological categories at the primary examination Biopsies from patients with diabetes mellitus, amyloidosis, systemic lupus erythematosus, periarteritis nodosa, and rheumatoid arthritis were also excluded as well as those from patients with a normal biopsy having none of the clinical signs of GN listed below The remaining biopsies were examined once more and classified according to histological type After classification of the biopsies, the clinical and laboratory data for each patient at the time of biopsy were collected from the hospital records The final series comprised 285 patients

Histological methods The biopsies had been fixed in either formaldehyde or glutaraldehyde Serial sections 2–3 µm thick were available for analysis and stained with

made

Criteria of histopathologic classification The classification used was based on light microscopic criteria reported by others (*Jones* 1957, *Ehrenreich & Churg* 1968, *Churg & al* 1970 *Habib* 1970, *White & al* 1970, *Burkholder* 1974, *Heptinstall* 1974)

A *Diffuse lesions* (all or most of the glomeruli affected)

Minimal change GN (MC) Glomeruli appear normal with no apparent cellular proliferation or thickening of the glomerular basement membrane (GBM) and no accentuation of the mesangial matrix No more than one out of 10 glomeruli is globally hyalinised

Menbranous GN (MGN) When stained with H&E, glomeruli show a general thickening of the capillary wall Staining with PASM reveals conspicuous needle like protrusions ('spikes') on the epithelial side of the GBM that occur either globally or segmentally in the glomeruli Mesangium is normal or only slightly increased with no cellular proliferation

Acute GN (AGN) A normal GBM is seen in combination with a proliferation of mesangial and endothelial cells that narrow or obliterate the capillary lumina Occasional polymorphonuclear leukocytes may be present in the glomeruli Mesangial matrix is normal

Mesangial sclerosis (mesangial proliferative) GN (MSGN) Capillary lumina are normal and patent Mesangial matrix is increased with or without proliferation of mesangial cells

Mesangiocapillary (membranoproliferative) GN (MCGN) The GBM appears double-contoured when stained with PASM and it may also stain strongly with PAS Mesangial matrix in increased, sometimes giving the glomeruli a lobulated appearance Proliferation of the mesangial cells is present The GBM may be only slightly affected but the prominent mesangial lobulation distinguishes MCGN from MSGN

Crescentic (rapidly progressive) GN (RPGN) Epithelial or fibro epithelial crescents appear within Bowman's space in most of the glomeruli, obliterating the glomerular tuft Unspecific proliferative changes are also seen within the tufts

B *Focal lesions* (only some glomeruli affected usually about a third)

Focal proliferative GN (FGN) Glomeruli show at least one of the following changes cellular proliferation increased mesangial matrix, capsular adhesion, sclerotic scarring Occasional crescents may be present as well as one or a few globally hyalinised glomeruli

Focal segmental glomerular sclerosis and hyalinosis (FSGSH) A segmental lesion in one or a few glomeruli shows hyaline material on the luminal side of the capillary wall Associated with this lesion may be foam cells, minimal cellular proliferation and some sclerosis with or without a capsular adhesion Some tubular atrophy is present

C *Unclassifiable proliferative GN* Glomerular changes are not compatible with any of the aforementioned types

Clinical and laboratory criteria

Persistent proteinuria more than 0 1 g/l of protein in the urine in repeated specimens

Haematuria three or more red blood cells per high power field in more than two samples of urine before biopsy

Nephrotic syndrome proteinuria in excess of 3 g per day in combination with at least two of the following oedema hypoproteinaemia (serum protein less than 55 g/l) or hypoalbuminaemia (serum albumin less than 30 g/l), and hypercholesterolaemia (serum cholesterol in excess of 7 5 µmol/l)

Renal insufficiency serum creatinine in excess of 110 µmol/l or a creatinine clearance less than 85 ml/min or both

Hypertension blood pressure higher than 150/100 mmHg regardless of age

Follow-up and calculation of survival Follow up time was defined as the period between the date of the first representative biopsy and the end of the study or death whichever came first The last day of the study was not fixed but was chosen to ensure, for all living patients a minimum follow up time of 7 years Death when not an actual death, was considered to have happened on the date when chronic haemodialysis was started or on the date of a renal transplantation if not preceded by dialysis (*Cameron* 1973, *Legrain* 1978) Survival data were collected from hospital records and from the official

TABLE 1 *Clinical Data*

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Focal segmental glomerular sclerosis & hyalinosis	3	2/1	35	28	27–50	1	1	–	–	–	1	–	–
Total series	285	154/131	37	34	15–73	74	56	91	54	42	55	76	7

* Number of patients

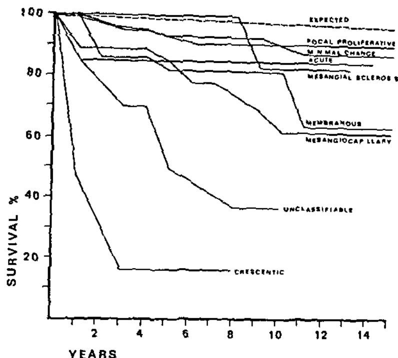


Fig 1 Actuarial survival according to histopathologic type of glomerulonephritis Expected survival calculated for total series

equally regardless of cause Expected survival (*Ederer & al* 1961) was calculated from mortality rate tables for the normal population for the period 1961–70 and the year 1977 kindly provided by the Finnish Central Bureau of Statistics Expected survival rates were adjusted for sex and age in each type of GN

Of the 285 patients seven were lost to follow-up (Table 1)

RESULTS

Clinical data (Table 1) More women than men had MC, MSGN, and FGN, mean age was highest among patients with RPGN, AGN, and unclassifiable GN No obvious correlations between clinical signs and the histopathologic type of GN could be found Of the patients with MC, only those with the nephrotic syndrome had received steroids In all other groups, some nephrotic and some non-nephrotic patients had been treated with steroids

[illegible] Expected survival was almost the same [illegible] *l* per [illegible] in of expected survival, therefore, the total series was pooled, all survival rates being compared with a single expected survival curve (Fig 1)

Survival was best in FGN and MC and progressively poorer in AGN, MSGN, MGN, MCGN unclassifiable, and RPGN (Fig 1) At 10 [illegible]

2) In RPGN, no ten year survival could be obtained In this group, however, a significant difference ($p<0\ 01$) from expected was found already after one year of follow-up No reliable survival curve could be calculated for the three patients with FSGSH Two of these patients survived with follow-up times of 8 1 and 14 8 years, respectively, the third patient died during the 5th year of follow-up

At ten years, the nephrotic syndrome correlated significantly ($p<0\ 01$) with mortality in patients

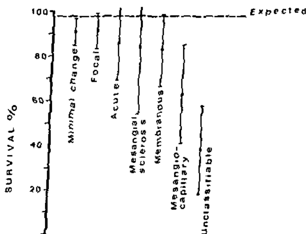


Fig 2 Ten year survival rates ± 2 SE according to histopathologic type of glomerulonephritis 10 year survival not obtained for crescentic glomerulonephritis (RPGN)

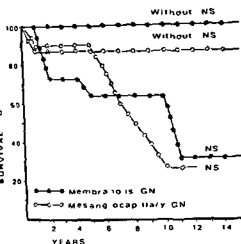


Fig 3 Effect of nephrotic syndrome (NS) on survival in membranous and mesangiocapillary glomerulonephritis

with *MGN* and MCGN (Fig 3) Survival did not differ, however, between nephrotic and non-nephrotic patients *with MC*

Fourteen patients with unclassifiable GN had some crescent formation in their biopsies Survival did not differ between these patients and the 13 whose biopsies showed no crescents

DISCUSSION

This study was undertaken to re-evaluate the prognostic value of the current light microscopic classification of idiopathic GN Immunofluorescence and electron microscopic findings were not taken into account Hence, some cases of MGN, because they looked normal on light microscopy (*Ehrenreich & Churg* 1968, *Bariéty & al* 1970, *Tornroth & al* 1978 *Tallqvist & al* 1979) may have been wrongly classified as MC As the short-term prognosis of this variety of MGN is good (*Tornroth & al* 1978), the effect of this source of error is probably minimal Electron microscopic studies have revealed two types of MCGN type I (subendothelial deposists) and type II (dense intramembarnous deposits) (*Bariéty & al* 1971 *Habib & al* 1973) That these types are not differentiated ultrastructurally in our study is probably of no consequence since actuarial survival is the same in both types (*Habib & al* 1975, *Davis & al* 1978)

Our results corroborate earlier observations that there is no consistent correlation between symptoms and histopathologic type of GN or *vice versa* (*Habib* 1970, *Cameron* 1975)

In our view, possible effects of steroid treatment on long-term survival cannot be analysed in retrospective studies The inclusion of treated as well as untreated patients in our study conforms with the practice in most other reports

As the occurrence of unrepresentative biopsies was random their exclusion from the study does not, in our opinion, affect the results

In the individual groups, survival was longest in MC and FGN and progressively shorter in AGN, MSGN, MGN, MCGN, unclassifiable GN, and RPGN For his comprehensive series *Cameron* (1972) reported no or minimal mortality in MC, FGN and MSGN [illegible]

[illegible]

Our survival curves show the same distribution for all types except MGN Among our patients with MGN, the survival rate was even higher than in MCGN, being about the same as that in AGN and MSGN

In MGN, survival was equal to that in the reports of *Legrain & al* (1978) and *Noel & al* (1979) That reports on survival in MGN differ may be due in part to variations in the frequency of the nephrotic syndrome (Table 2) Our results show, in agreement with *Habib & al* (1973 a) and *Legrain & al* (1978) that in MGN the nephrotic syndrome has a negative influence on prognosis It may be, however, that in a series in which a high percentage of patients with MGN are nephrotic, biopsies have been obtained only from those with severe and longstanding disease – which might affect the mortality rate *Beregi & Varga* (1974) performed biopsies on patients with MGN soom after the onset of clinical signs, and the frequency of the nephrotic syndrome in their series was very low (Table 2) But the relation of the nephrotic syndrome to survival in MGN is not without controversy in two of the aforementioned studies (*Cameron* 1972, *Noel & al* 1979) [illegible]

[illegible] Our survival results agreed with those in all earlier studies (*Antoine & Faye* 1972, *Cameron* 1972, *Cameron* 1973, *Cameron & al* 1973, *Habib & al* 1973, *Habib & al* 1975, *Barbiano di Belgiojoso & al* 1977, *Davis & al* 1978, *Legrain & al* 1978) That the nephrotic syndrome affected mortality in MCGN agrees with earlier reports (*Barbiano di Belgiojoso & al* 1977, *Davis & al* 1979 [illegible]

Wi [illegible] those with 'other proliferative GN' (*Cameron* 1972) The high mortality in unclassifiable GN can partly be explained by the finding that half of the biopsies

TABLE 2 *Frequency of Nephrotic Syndrome (NS) Reported in Membranous Glomerulonephritis*

Reference	Patients with NS/all patients	% with NS
Forland & Spargo 1969	19/19	100
Hardwicke & al 1967	40/43	93
Gluck & al 1973	35/38	92
Habib 1970	37/41	90
Habib & al 1973 a	44/50	88
Erwin & al 1973	41/48	85
Row & al 1975	54/66	82
Pollak & al 1968	17/21	81
Cameron 1975	53/69	77
Noel & al 1979	84/116	76
Cameron 1972	30/41	73
Ehrenreich & Churg 1968	44/60	73
Franklin & al 1973	21/32	66
Legrain & al 1978	53/81	65
Beregi & Varga 1974	20/260	8
Present study	11/22	50

showed some crescent formation The number of crescents has been shown to correlate with survival regardless of type of GN (*Zollinger & Mihatsch* 1978) In our unclassifiable GN patients mortality was high even in the absence of crescents However capsular adhesions were found in all of the unclassifiable biopsies in combination with varying proportions of hyalinised glomeruli Adhesions are thought to represent healed crescents (*Zollinger & Mihatsch* 1978) Our findings indicate that such lesions may represent a prognostic sign equal in importance to crescent formation

FSGSH has generally been reported to be associated with a poor actuarial survival (*Cameron* 1972, *Cameron & al* 1978 *Legrain & al* 1978) Our series contained too few patients with FSGSH for any valid observation Reported frequencies of FSGSH have been variable ranging from 10 4 (*Habib* 1970) and 6 5 per cent (*Cameron* 1972) to 1 6 (*Velosa & al* 1975) and 1 3 per cent (*van Acker & al* 1975) The low frequency in our series cannot be explained by the misdiagnosis of FSGSH as MC or FGN since this would probably have affected the survival rate in these groups

Conclusion Our results show that the prognosis of GN is fairly good in most types The only exceptions were MCGN unclassifiable GN and RPGN which differed significantly from expected The nephrotic syndrome was found to worsen prognosis in MGN and MCGN, but not in MC In contrast to conclusions drawn by others (*Cameron* 1972 *Habib* 1975) but in agreement with *Cameron* (1975) we conclude that the current light microscopic classification of GN is only a relatively crude guide to prognosis Clinical findings such as the nephrotic syndrome, must also be accounted for

We are grateful for the technical assistance of Miss *Riitta Liisa Laine* and Miss *Riitta Liimanen* This study was supported by a grant from the *Sigrid Juselius Foundation*

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THE FINE STRUCTURE OF »DIVIDERS« AND »NON-DIVIDERS« IN PHASE II HUMAN GLIAL CELL CULTURES

E BLOMQUIST[1] E ARRO[1] U BRUNK[1] V P COLLINS[2] and B A FREDRIKSSON[1]

Institute of Pathology[1] University of Uppsala Uppsala and Department of Tumor Pathology[2] Institute of Pathology Karolinska Institute Stockholm Sweden

Blomquist, E Arro E Brunk U Collins V P & Fredriksson B A The fine structure of »dividers« and »non-dividers« in phase II human glial cell cultures Acta path microbiol scand Sect A 88 327–337 1980

A method is described which allows a comparison in the transmission electron microscope (TEM) of cells with different remaining proliferative capacity from one and the same culture The method takes advantage of a mini-cloning technique employing haptotactic palladium islands in combination with micro dissection and preparation for TEM of islands carrying various numbers of cells after 10 days in culture when all miniclones have become density dependent growth inhibited By means of this technique non-dividers were compared with miniclones of dividers composed of five to eight cells originating from single cells Moreover large immotile cells without peripheral ruffling activity known to be non-dividers were compared with small ruffling cells known to be dividers in the reflection interference mode in sparse cultures of living cells and in the TEM mode as whole cell preparations after critical point drying of cells cultured on formvar-coated gold EM grids Non dividers proved to contain a moderate number of residual bodies well developed Golgi areas and often branched or circular mitochondria they were thinly spread over the substratum with many focal points of contact and large areas of close apposition between cell and substratum

Key words Glial cell cultures ultrastructure

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In recent years it has been clearly shown that any passage of cultured diploid normal cells will include those with proliferative capacity and those with little or no such potential (*Merz & Ross* 1969 *Cristofalo & Sharf* 1973 *Smith & Hayflick* 1974)

As def[illegible] d ploid [illegible] be divi[illegible] III (*Ui*[illegible]) During phase I the cells are established *in vitro* in phase II they grow exponentially and in phase III the cultures become impossible to subcultivate due to insufficient proliferation Normal human glial cells behave in this manner

[illegible] to study the relationship between cellular proliferative capacity under various defined conditions and passages of varying age (*Blomquist et al* 1978 *Blomquist et al* 1979 *Collins et al* 1979a)

The human glial cell line U 787 CG can be cultured for a maximum of 48 passages After about 30 passages the proportion of cells which do not divide within 10 days after subcultivation onto palladium squares on agarose (classified as non dividers) is 40% while the cells which form mini clones of more than four cells on the haptotactic

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As defined by Hayflick the normal life span of a diploid non transformed cell population *in vitro* can be divided into three periods termed phases I II and III (*Hayflick & Moorhead* 1961) During phase I the cells are established *in vitro* in phase II they grow exponentially and in phase III the cultures become impossible to subcultivate due to insufficient proliferation Normal human glial cells behave in this manner

In earlier studies of cultured glial cells we used a mini-cloning system of small palladium squares on agarose (*Westermark* 1978 *Blomquist et al* 1980) to study the relationship between cellular proliferative capacity under various defined conditions and passages of varying age (*Blomquist et al* 1978, *Blomquist et al* 1979 *Collins et al* 1979a)

The human glial cell line, U-787 CG can be cultured for a maximum of 48 passages After about 30 passages the proportion of cells which do not divide within 10 days after subcultivation onto palladium squares on agarose (classified as non dividers) is 40% while the cells which form mini clones of more than four cells on the haptotactic

islands number only 10% (*Collins et al* 1979a, *Blomquist et al* 1980)

The non-dividers of this system present a characteristic morphology They are large, widely and thinly spread and show almost no outward signs of motility in the form of lamella formation or ruffling along their periphery, nor do they translocate (*Blomquist et al* 1978, 1979)

This is in contrast to cells with persisting proliferative capacity, such cells are smaller, less well spread and grow more densely (*Blomquist et al* 1978, *Collins et al* 1979a, 1979b)

So far, it has not been possible to compare known non-dividers with cells retaining proliferative capacity (dividers) in the transmission electron microscope using sectioned material, the proliferative history of individual cells being unknown in ordinary mass cultures We here describe the application of the haptotactic island system (*Carter* 1967, *Harris* 1973, *Westermark* 1978) to make this possible

The causes of the phase III phenomenon are at present unknown although diverse hypotheses have been advanced (*Hayflick* 1977) A detailed study of the morphology of cells which have exhausted their proliferative capacity, together with a comparison with cells from the same culture and passage which have shown proliferative capacity, might give some clues to the mechanisms underlying this phenomenon

MATERIAL AND METHODS

Cell Culture

The experiments were carried out on the glial cell line U 787 CG The cells were cultured as previously reported (*Ponten et al* 1969) They were grown in Eagle s minimal essential medium supplemented with antibiotics and 10% fetal calf serum in Nunclon® or Falcon® plastic Petri dishes Cells from passage 30 were subcultivated into plastic dishes with haptotactic islands as described below or into dishes containing carbon stabilized formvar covered EM gold grids as described by *Wolosewick and Porter* (1976) The haptotactic island cultures were harvested for EM after 10 days and the gold grid cultures after 3–4 days

Preparation of Haptotactic Islands

Haptotactic islands (7 056 μm² squares) were deposited on a layer of 1% agarose as previously described in detail (*Blomquist et al* 1978) The islands were formed with the help of standard (200 mesh) copper grids for electron microscopy

Phase-Contrast and Interference Reflection Microscopy

Cells were seeded out on 25 mm round cover slips After two days the cover slips were mounted in a Dvorak Stotler chamber filled with pH controlled culture medium Sparse cultures of living cells examined and photographed at 37 °C in phase-con[illegible] and interference reflection modes using Kodak ASA Plus-X-film

The interference reflection microscope (Leitz Dia[illegible] is essentially a standard reflected light micros[illegible] illuminated by linearly polarized light (50 W merc[illegible] light source) with a crossed analyzer in the view[illegible] optics for the reduction of internal reflections in [illegible] microscope The key accessory is a lambda/4 p[illegible] between the objective lens and the object (combined the new Leitz Pl Apo 40/1 00 immersion objecti[illegible] which circularly polarizes the light reflected from [illegible] object to pass the analyzer in the viewing optics

Transmission Electron Microscopy

A *Cells on haptotactic islands* The cells were fixed [illegible]

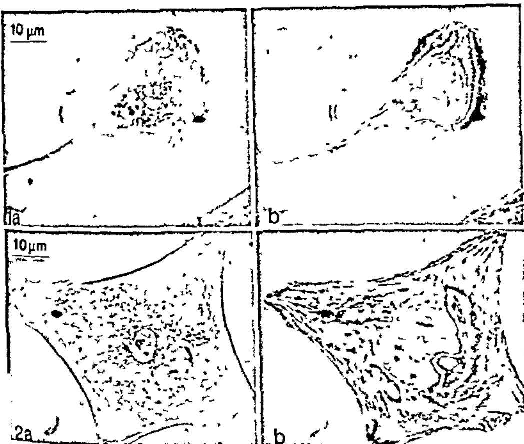
then at 4 °C for one hour The medium covering [illegible] haptotactic islands was changed for the fixative witho[illegible] any previous rinse About 800 palladium squares w[illegible] large, single cells (non dividers after 10 days) w[illegible] individually removed from the agarose by means o[illegible] thin tungsten needle under a dissecting microscope a[illegible] transferred to a Beem capsule containing glutaraldehy[illegible] fixative Likewise about 500 palladium squares ea[illegible] with miniclones consisting of 5–8 cells were collected [illegible] another Beem capsule After a brief rinse in 0 15 M N[illegible] cacodylate buffer the preparations were post fixed in 1[illegible] OsO_4 in 0 15 M Na cacodylate buffer for 90 min [illegible] room temperature The further processing includi[illegible] dehydration and embedding into Epon 812 was carri[illegible] out in the same capsules During dehydration the cel[illegible] were impregnated for 12 h in 1% uranyl acetate in 50[illegible] ethanol The islands were spun down in a Beckman TJ [illegible] centrifuge between the changes of solution Aft[illegible] polymerization the blocks were cut with a diamon[illegible] knife on an LKB Ultrotome V The thin sections wer[illegible] collected on formvar covered grids stained with lea[illegible] citrate and examined in a Jeol 100 C electron microscope

B *Whole cells on gold grids* The gold grids wit[illegible] cultured whole cells were fixed dehydrated and critica[illegible] point dried as previously described (*Blomquist et al* [illegible] [illegible] impregnated [illegible] The [illegible] scope equipped with a side entry goniometer Stereo pairs were photographed at various angles up to 20 °C depending on the magnification

RESULTS

Phase-Contrast and Interference-Reflection Microscopy of Living Cells

The cultures contained a considerable number of large, well spread and immobile cells which have been shown in previous studies to be non-dividers



Figs 1A and 1B Small motile cell of the »dividing« type showing a leading lamella with ruffling activity In the phase-contrast mode (IA) the thicker perinuclear area containing organelles can easily be differentiated from the peripheral thinner well spread cytoplasm Note the relatively few small dark areas (arrows mark some of them) in relation to the outer gray zones in the reflection interference mode (IB) indicating focal contacts between the cell and its supporting substratum The gray zones indicate areas with close apposition between the cell and its substratum

Figs 2A and 2B Large immotile cell of the »non-dividing« type showing an almost carpet like distribution of stress fibres in the phase-contrast mode (2A) and multiple focal contacts between the cell and its substratum (arrows) in the reflection interference mode (2B) The gray zones (areas of close apposition) are large and irregular The inner circular zones may represent secondary reflection from within the cell

(Blomquist et al 1978 Collins & Willems 1980) They were intermingled with apparently smaller and less well spread cells presenting a leading edge with ruffling activity a cell form and behaviour demonstrated previously to be associated with entry into and progression through the cell cycle (Brunk et al 1976) In the sparse cultures it was easy to discriminate between these cell types and select typical representatives

The living cells selected for photography were first identified and photographed in the phase contrast mode and the same cells then immediately studied in the interference reflection mode Vigor-

ously moving cells showed concentric rings of gray areas which were interpreted as areas of close contact (about 30 nm) between the cell and the supporting glass (Fig 1 A and 1 B) However the innermost rings may also result from secondary and higher order of reflections obtained from structures within the cytoplasm or the upper cell surface (Abercrombie & Dunn 1975 Issard & Lochner 1976)

In addition to peripheral gray regions (outer concen-

islands number only 10% (*Collins et al* 1979a *Blomquist et al* 1980)

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Transmission Electron Microscopy

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room temperature The further processing including dehydration and embedding into Epon 812 was carried out in the same capsules During dehydration the cells were impregnated for 12 h in 1% uranyl acetate in 50% ethanol The islands were spun down in a Beckman TJ 6 centrifuge between the changes of solution After polymerization the blocks were cut with a diamond knife on an LKB Ultrotome V The thin sections were collected on formvar covered grids stained with lead citrate and examined in a Jeol 100 C electron micro scope

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2 µm
1
4

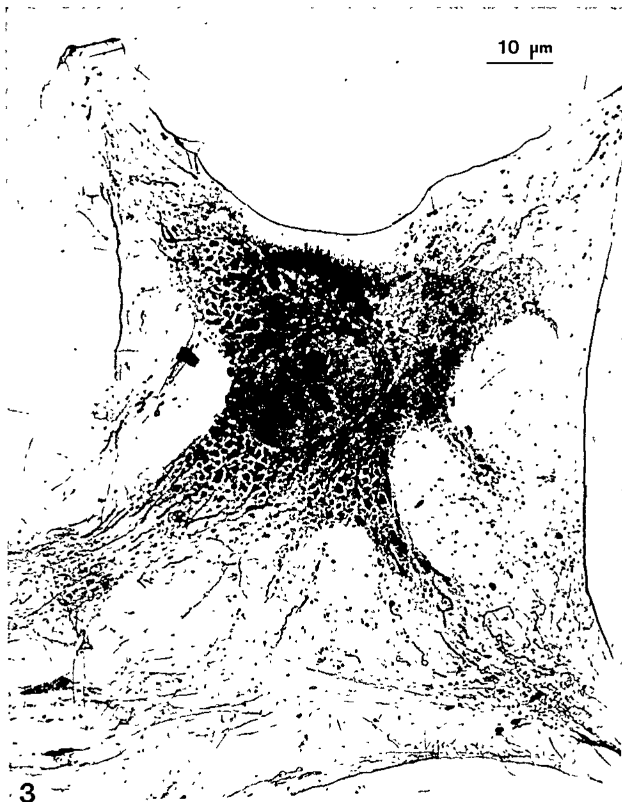


Fig 3 Composite of eight different negatives showing a »non-divider« Note the rather limited, dense and thick perinuclear region containing the majority of mitochondria and residual bodies which contrasts with the large, thinly spread peripheral cytoplasm containing many bundles of microfilaments and relatively few mitochondria and residual bodies

... many residual bodies
...air in Fig 7

Fig 5 Well spread part of a non-divider showing the degree of irregular ... relation between mitochondria and the electron dense residual bodies


2 µm

5 μm

6



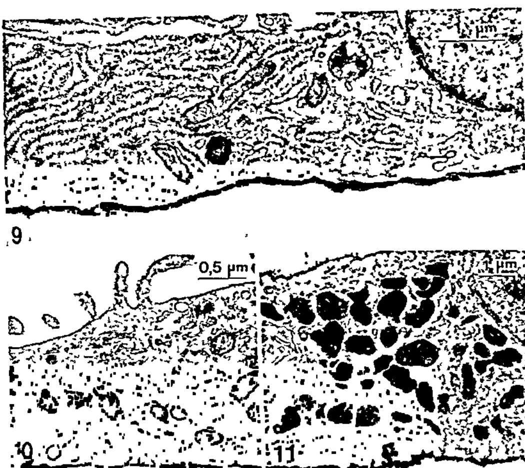


Fig 6 Peripheral thin part of a non-divider with multiple bundles of microfilaments

Fig 7 Stereo pair (± 6°) of a detail from Fig 4 with a circular mitochondrion enclosing a residual body Note tiny filaments appearing to connect the residual body with the mitochondrion Such filaments are seen around many of the free residual bodies too and also along the periphery of the mitochondria It is not known whether these filaments are real or represent artefacts formed during the inevitable cellprotein transformation during fixation dehydration and critical point drying

Fig 8 Parts of three cells in a miniclone (cells retaining dividing capacity) The palladium substratum is arrowed A specialized area of close contact between the cell and the surface is indicated (arrow heads) Note pinocytotic vesicles originating from the cell surfaces mainly the lower

Fig 9 Detail from a cell in a miniclone showing part of the nucleus endoplasmic reticulum mitochondria and few residual bodies

Figs 10 and 11 A divider and a non-divider Note the difference in the number of residual bodies Both cells show the perinuclear area The divider has a few microvilli along its upper cell surface and shows evidence of pinocytotic activity (arrow)

of focal contact (10–15 nm) between the cells and the glass surface (*Curtis* 1964 *Abercrombie & Dunn* 1975 *Izzard & Lochner* 1976 *Heath & Dunn* 1978) The perinuclear part was in general light gray to white indicating a region of higher separation (more than 100 nm) between the cell and the glass surface

The large immobile and widely spread cells were well endowed with stress fibres (Fig 2 A) and had a fairly thin peripheral zone outside the perinuclear area. In the interference reflection mode it was seen that the gray zones were far more pronounced broader and less regularly arranged than in vigorously moving cells (Fig 2 B) It is unknown

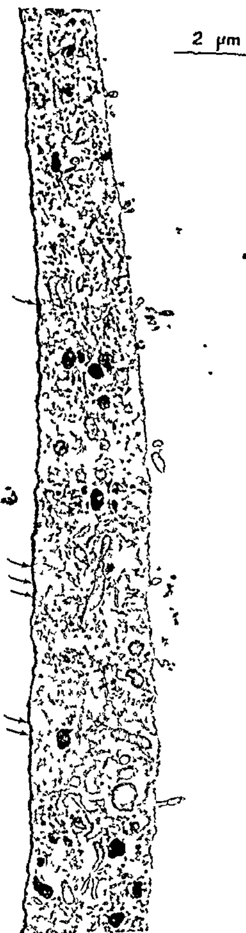


Fig 12 Composite of four micrographs to show a major part of a *non divider* Note the extent of the cell and the adherence to the substratum Groups of pinocytotic vacuoles are *forming (arrows) showing that* some capacity for endocytosis apparently persists al though the cells lie very close to the substratum

whether the inner, circular gray areas represent zones of close apposition between the cells and the substratum or are due to secondary reflection within the cytoplasm The immobile cells also showed many black areas representing focal contacts between the cell and the substratum usually in relation to the stress fibres and mostly situated at the periphery of the cells

Ultrastructure of Whole Cells Grown on Gold Grids
The small cells presented the morphology pre viously described (*Collins et al* 1978) Large well spread immobile cells were readily identified on the carbon formvar coated gold EM grids In low magnification (*Fig 3*) these cells had a highly characteristic morphology with a comparatively thin perinuclear zone occupied by mitochondria and numerous irregularly shaped electron dense orga nelles outside which the cell was very thinly spread and the cytoplasm contained a few mitochondria of various shapes and sizes as well as bundles of microfilaments (stress fibres) At higher magnifica tions the morphology of the mitochondria could be studied in detail (Figs 4 and 5) They were often ramified or circular findings which cannot be easily demonstrated in randomly orientated sections as the mitochondria with their branches usually lie hori zontal to the substratum and sections in such a plane are understandably few In the thinly spread area peripheral to the perinuclear zone the cells also contained a number of electron dense irregular organelles resembling residual bodies (Fig 4) some of which showed an intimate relation to mitochon dria being occasionally located within a ring shaped part of a mitochondrion as shown in detail in a stereo pair micrograph (Fig 7) In such cases a number of tiny filaments were seen to bridge the gap between the residual body like structure and the inner circumference of the mitochondrion Other non mitochondria related residual bodies were also surrounded by tiny filaments For the time being it is hard to say whether these filaments are real structures e g related to the microtrabecular network as described by Wolosewick and Porter (1979) or artefacts created during the critical point drying of the cells The most peripheral regions of the cell were mainly occupied by microfilaments

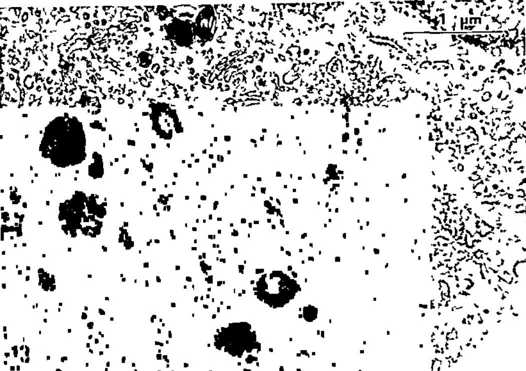


Fig 13 Perinuclear zone of a non divider showing well developed Golgi areas along with smooth and rough endoplasmic reticulum and residual bodies

which were evenly distributed in mat like arrays or arranged in bundles (Fig 6)

Transmission Electron Microscopy of Ultrathin Sections from Embedded Cells

a Miniclones consisting of 5–8 cells The cells showed the same morphological features as have already been described in studies of density dependent growth inhibited cultures from early passages (*Brunk et al* 1973 *Collins et al* 1979c) The cells were essentially monolayered had few residual bodies and a well developed endoplasmic reticulum The Golgi zones were well developed with emergent coated vesicles Autophagosomes occurred occasionally Micropinocytotic vesicles were chiefly associated with the lower cell surface Occasional ruffles were registered at the cell borders (Figs 8 9 and 10)

b Haptotactic islands containing single cells only (non dividers at 10 days) The non-dividers were large and flat and had few microvilli Occasionally small lamellae could be found at the cell periphery The endoplasmic reticulum appeared to be less well developed than in the multiplying cells The Golgi zones however were large and surrounded by many small vesicles both smooth surfaced and coated The residual bodies were relatively numerous but far fewer than found in cells which have been kept in a stable monolayer for several weeks (*Brunk et al* 1973 *Collins & Brunk* 1978) Micropinocytotic vesicles were present both at the upper and the lower surface although less frequently than in the case of the dividers The cells appeared to adhere closely to the palladium substratum which may restrict the uptake of nutrients by pinocytosis from the lower cell surface (Figs 11 12 and 13)

DISCUSSION

The minicloning technique utilizing haptotactic palladium islands and the subsequent registration during a 10 day period of the potential for proliferative activity of initially single cells under standard culture conditions makes it possible to discriminate between cells lacking proliferative ability and cells retaining the capacity for division (ability to form miniclones of 5 to 8 cells on the 7 056µm² palladium islands) The combination of this technique with micro-dissection and collection

for embedding in resin and eventual electron microscopy of islands carrying these two types of cells allows us to compare their fine structure

The method described offers an opportunity to analyze populations of cells with defined prolifera-tive or other abilities and known history, and to discriminate between groups of such cells in a way not possible with mass cultures The findings in morphological studies of the present type should preferably be analyzed with morphometric techni-ques Due to the rather complicated and time-consuming procedures employed it was deemed justifiable to limit the present paper to a description of the preparative technique to indicate its useful-ness, and to include an account of some of the major differences between dividers and non dividers Detailed analyses of e g differences in pinocytotic activity between the two groups of cells require stereology and statistical evaluation

In a mass culture non-dividers can be identified by their large size and the fact that they are well spread without the ruffling lamellas characteristic of [illegible] cells can be identified [illegible]

in the TEM They cannot, in such preparations, be compared with the daughter cells of cells which have shown a large proliferative capacity despite the limitation of the factors which are required for cellular proliferation – as is the case in the miniclone system where 5–8 cells have resulted from proliferation on a 7,056 μm² island under otherwise standard conditions

The most noticeable difference between dividers and non dividers was that the latter, apart from their size, spreading capacity and immobility, contained a highly developed network of microfila-ments, often matted but sometimes in the form of stress fibres The findings are not unlike those obtained when serum starved phase II glial cells (which are blocked in G_1 and also immobile) (*Collins et al* 1978) are examined The non dividers obviously expand and spread maximally This is reflected both in the relatively sparse micropinocy totic activity from their lower cell surface and in their reflection-interference pattern showing large areas in close contact with the supporting substra tum Earlier experiments have shown that cells manifest increasing demands for both supporting areas and growth promoting factors in order to be able to divide as they approach phase III and that these parameters can, within certain limits, compen-sate one another (*Blomquist et al* 1979, *Collins et al* 1979a) It thus seems probable that glial cells become increasingly resistant during continued passage to the aforementioned growth promoting stimulation, and demand increasing amounts of the necessary factors in order to be able to enter the cell cycle

The non-dividers showed a somewhat less well developed endoplasmic reticulum, and more nume-rous large, branched and partially circular mito chondria, than the dividers Similar observations have been made in phase II glial cells which were growth inhibited by serum starvation (*Collins et al* 1978)

The increasing number of residual bodies in the non-dividers would seem to be merely a reflection of their non-dividing state, since residual bodies rapidly accumulate even in phase II cells if they are blocked in G_1 for long periods (*Brunk et al* 1973, *Collins & Brunk* 1978) It should be stressed that the non-dividers never contained as many residual bodies as dividers regularly do after some weeks in a non-dividing state (*Collins & Brunk* 1978) Accor-dingly, the investigated non-dividers had probably been blocked in G_1 only for a comparatively short time when they were studied This favours the hypothesis that non-dividers are fragile and rarely survive the somewhat rough subcultivation treat-ment (*Milo* 1973) We therefore believe that most of the non-dividers studied became incapable of division probably in relation to the last subcultiva tion

The phase III phenomenon – typical of all normal diploid cells which do not transform spontaneously – apparently arises because an increasing fraction of cells in the cultures lose their dividing capacity in the higher passages (*Cristofalo & Sharf* 1973, *Blomquist et al* 1980) This has been termed »degeneration« or even »terminal differentiation« (*Hayflick* 1977, *Bell et al* 1978, *Kontermann & Bayreuther* 1979) Since the non-dividers can survive for a considerable time and at least some of them clearly are only »relative non-dividers« – they may be induced to reenter the cell cycle by an increase in growth promoting factors or substratum area (*Blomquist et al* 1979, *Collins et al* 1979a) – the relationship between the phase III phenomenon and ageing in the complex multicellular organism may be questioned On theory the phase III phenomenon represents a form of cell growth control Such a control would prevent unrestricted neoplastic growth by limiting the number of daughter cells which a cell can provoke

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Supported by grants from the *Swedish Medical Research Council* and the *Swedish Cancer Society*

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Acta path microbiol scand Sect A 88 339-340 1980

BRIEF REPORT

STAINING OF RAT THYROID PARAFOLLICULAR (C-) CELLS WITH THE SEVIER MUNGER SILVER TECHNIQUE

Erik Wilander Lisa Juntti Berggren Monalill Lundqvist and Lars Grimelius

Department of Pathology University of Uppsala Uppsala Sweden

Wilander E Juntti Berggren L Lundqvist M & Grimelius L Staining of rat thyroid parafollicular (C-) cells with the Sevier Munger silver technique Acta path microbiol scand Sect A 88 339-340 1980

The argyrophil silver stain of Grimelius and of Sevier Munger was studied in rat thyroid parafollicular (C-) cells after formalin and Bouin fixation The strongest and most easily reproducible staining reaction was obtained in tissues fixed with Bouin s fluid and stained by the Sevier Munger technique The identity of the argyrophil cells as the calcitonin containing C-cells was established by re staining the same sections with calcitonin antibodies according to the method of Sternberger

Key words Thyroid parafollicular C-cells silver staining technique

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Received 7 iii 80 Accepted 12 vi 80

Silver stains are often used to identify different types of endocrine cells The usefulness of these methods however is dependent on the examiner s knowledge of the reaction of the individual endocrine cell types to each silver stain In the present report an argyrophil reaction with the method of Sevier Munger is demonstrated in parafollicular cells of the rat thyroid The identity of these cells as the calcitonincontaining C-cells was established by re staining the same sections with calcitonin antibodies according to the indirect immuno histochemical (PAP) method of *Sternberger* (5)

Materials and methods

Six adult male albino rats (body weight about 250 g) of the Sprague Dawley strain obtained from Anticimex AB Stockholm were used Under general anaesthesia induced with ether the thyroid gland was carefully removed fixed in Bouin s fluid or 10% formalin for 24 hours dehydrated and embedded in paraffin Sections about 4 m th k [illegible]

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displayed a stronger and more reproducible argyrophil reaction in scattered mostly parafollicular cells than those stained by the method of Grimelius In the formalin fixed tissues the argyrophil reactions were weak or negative Areas of the Bouinfixed and Sevier Munger stained sections were photographed at a magnification of × 250 and then de stained with 1% KCN in aqueous solution (2) for about 30 min The sections were [illegible]

in the sections as before were photographed again at the same magnification The calcitonin antibodies employed (a gift from Dr *G Lundqvist*) were raised in rabbits after conjugation of synthetic calcitonin to bovine serum albumin For the present experiments the antibodies were diluted 1 100 Controls as recommended by *Goldberg* (1) were used

[illegible]

Sevier Munger technique (Fig 1) These cells were [illegible] tissue [illegible] against [illegible] After [illegible] apparent that the argyrophil cells were identical to the cells showing calcitonin like immuno-reactivity although the intensity of the staining reaction varied to

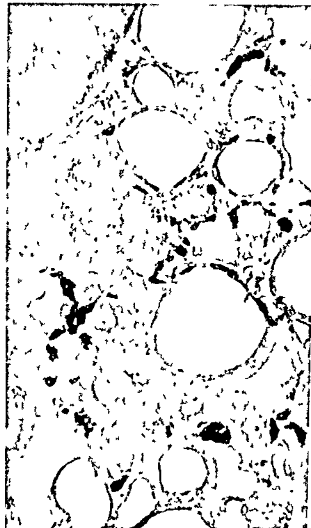


Fig 1 Light microscopic photograph of the rat thyroid gland Parafollicular cells with heavy black cytoplasmic granulation are seen Sevier Munger silver stain × 250



Fig 2 Photograph of the same area as in Fig 1 after restaining with calcitonin antibodies The Sevier Munger stained cells seen in Fig 1 correspond comple tely to the cells showing calcitonin immunoreactivity PAP technique × 250

some extent between individual cells (Fig 2) The control sections were negative

The results show that in Bouin fixed rat thyroid tissue both the argyrophil technique of Sevier Munger and that of Grimelius can be used to identify the calcitonin containing parafollicular cells of the rat thyroid With the former method however the parafollicular cells are more sharply outlined and more reproducible results can be obtained in quantitative studies for instance

Although immuno cytochemistry with specific antibo dies is the most accurate method for visualizing a particular type of endocrine cell silver stains are often used as well because 1) antibodies are sometimes expensive or difficult to obtain 2) the hormone content of some endocrine cell types is unknown and 3) the argyrophil silver techniques are nondiscriminating and stain several types of endocrine cells (3) and can thus be utilized as screening methods for these cells

This work was supported by grants from the *Swedish Medical Research Council* (Project No 102)

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Acta path microbiol scand Sect A 88 341–353 1980

STUDIES ON THE RAT LIVER FOLLOWING IRON OVERLOAD

Electron Microscopical and Histochemical Investigation after Iron Depletion

ROLF HULTCRANTZ[1] BENGT ARBORGH[1] ROMULAD WROBLEWSKI[2] and JAN L E ERICSSON[3]

[1]Department of Pathology Karolinska Institutet Huddinge Hospital S 141 86 Huddinge [2]Wenner Gren Institute University of Stockholm Stockholm and [3]Department of Pathology University of Uppsala Uppsala Sweden

Hultcrantz R Arborgh B Wroblewski R & Ericsson J L E Studies on the rat liver following iron overload Electron microscopical and histochemical investigation after iron depletion Acta path microbiol scand Sect. A 88 341–353 1980

Sprague Dawley rats were given Jectofer injections to obtain iron overload in rat liver Kupffer and parenchymal cells The release of iron was studied on the light and electron microscopical level by bleeding of the

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was demonstrated with X ray microanalysis at the ultrastructural level and presence of acid phosphatase was revealed using a histochemical method based on precipitation of lead phosphate at sites of enzyme activity in combination with X ray microanalysis It was demonstrated that all IP containing cytoplasmic bodies also showed presence of acid phosphatase implying that all of them represented lysosomes The mechanism of release of iron from Kupffer and parenchymal cells was discussed with special emphasis on the role of the lysosomes and the relationship between their contents and other cellular structures

Key words Liver iron overload ultrastructure histochemistry

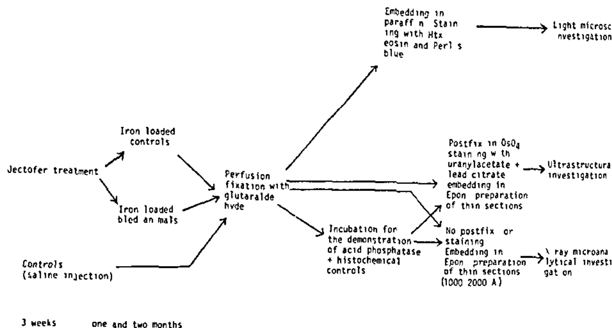
Rolf Hultcrantz Department of Pathology Huddinge Hospital S 141 86 Huddinge Sweden

Accepted as submitted 2 v 80

Studies on iron overload in experimental animals have clarified that a substantial part of the excessive iron is stored in the liver (23 1 26 10 20) Considerable interest has been focused on the processes of uptake and storage (26 20 6 19, 24 16 17) In our previous studies we have described the ultrasturctural and histochemical basis for the handling of excess iron in the rat liver with special reference to the role of the lysosomal vacuome (13 14) On the basis of this knowledge it has [illegible] pholo-gic expressions of turnover metabolism and release of iron in the overloaded liver

In order to study the mobilization of iron from the overloaded liver rats have been bled repeatedly whereby the demand for iron for synthetic purposes in the animals has been greatly enhanced By this means an experimental model useful to study if and how different types of liver cells are able to release iron has been created It has further been possible to clarify whether or not different types of lysosomes in these cells react in the same way in a situation of iron demand outside the liver

Table 1 Short Survey over the Handling of the Investigated Material



MATERIALS AND METHODS

For a short survey see Table 1

Iron Loading

Male Sprague Dawley rats (weighing 200 220 grams) maintaned on ordinary diet consisting of pellets contai ning approximately 260 μg iron/g (R3 Astra Evos Sodertalje Sweden) and water *ad lib* were given intramuscular injections of a citric acid iron sorbitol complex (Jectofer® Astra Pharmaceutical Company Sodertalje Sweden) 15 times during a three week period the total dose corresponding to 50 mg Fe^{+++} per 100 g of body weight (these rats are referred to in the following as *iron loaded animals*) *Control rats* were given intramuscular injections of saline

Mobilization of Iron from the Liver

After completed injections of iron the rats were divided into two groups each containing 10 animals One group served as control and received no further treatments The rats in this group are henceforth referred to as *iron loaded non bled animals* Thus they constituted the *iron loaded control group* The experi mental group *iron loaded bled animals* was subjected to weekly phlebotomizations in which 1 5 ml peripheral blood/100 g of body weight (corresponding to ~25% of the total blood volume) were withdrawn by punctu ring the orbital plexus with a heparinized glass capillary under slight ether anesthesia Measurements of the [illegible] weekly on [illegible] the tail In [illegible] globin was [illegible] measured immediately before phlebotomization The animals were sacrificed *1* and *2* months after the start of the phlebotomization along with iron loaded controls and the control animals only given saline

Fixation

All livers were fixed by perfusion The abdomen w cut open during ether anesthesia and a catheter w inserted into the portal vein and ligated The infer caval vein was opened above and below the liv

buffer containing 0 1 M sucrose as described previous (13) The liver tissue was cut into 1 mm thick cubes ar slices and was fixed for 24 hours in the fixati mentioned above

Preparation for Microscopy

Tissues for light microscopy were embedded i paraffin and stained with hematoxylin eosin and Perl blue

The material intended for *electron microscopy* wa postfixed in 2% s collidine buffered osmium tetroxid and embedded in Epon after dehydration in ethanol and propylene oxide *En bloc* staining with 1% uranyl acetate was performed Ultrathin sections were prepared on a LKB ultrotome V stained with lead citrate and examined in a Jeol 100 C electron microscope

Demonstration of Acid Phosphatase

The slices fixed in glutaraldehyde for 24 hours were rinsed in 0 1% sucrose in 0 1% cacodylate buffer immersed in 10% DMSO 0 1 M cacodylate buffer and 0 1 M sucrose at pH 7 2 for 24 hours and were then cut into 50 μm thick sections using a Leitz freezing microtome These sections were incubated for 15 45 60 or 90 minutes in a Gomori type medium (2) containing

14% sucrose and were subsequently rinsed in 0 1 M cacodylate buffer Controls were incubated in media

described above Ultrathin sections were cut on an LKB ultrotome V stained with lead citrate and examined in a Jeol 100 C microscope

X ray Microanalysis

For X ray microanalysis glutaraldehyde fixed tissues (either as such or incubated for the demonstration of acid phosphatase) were cut in 100–200 nm thick sections which were placed on nylon grids No postfixation in osmium staining with uranyl acetate or lead citrate was carried out on this material Analytical electron microscopy was performed with a Kevex energy dispersive X ray spectrometer in a Jeol C electron microscope provided with a Jeol ASID scanning attachment (14) The tissue was examined in the scanning transmission mode at 100 KV using a take-off angle at 30° The counting time was 50 sec (14)

RESULTS

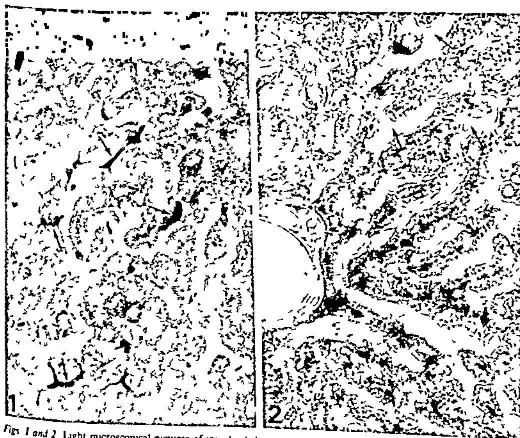
Hemoglobin measurements Following the bleeding, hemoglobin concentration of the blood decreased 10–15% The hemoglobin concentration in the non bled animals ranged between 155–160 gram hemoglobin/liter

MORPHOLOGY

Light Microscopy

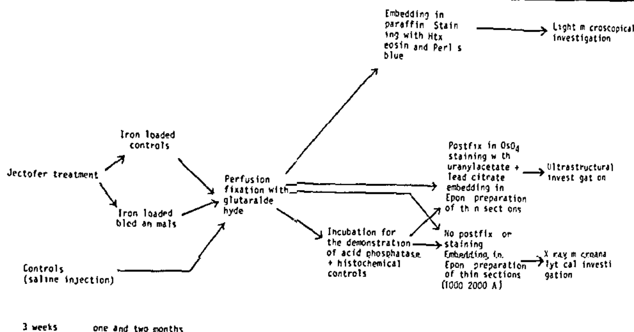
No pathological changes other than those directly dependent on the iron overload (described below) could be demonstrated in the liver tissue from any of the animals

Iron loaded animals Following completed injec-



Figs 1 and 2 Light microscopical pictures of iron loaded rat liver tiss ... sections stained for the demonstration of iron In *Figure 1* ... iron positive pigment in parenchymal cells Iron positive ... (arrows) In *Figure 2* – showing tissue from a bled animal ... (arrows) However in the parenchymal cells the same inte... cells in Figure 1 present Glutaraldehyde Perl s blue × 2...

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Fixation

All livers were fixed by perfusion The abdomen was cut open during ether anesthesia and a catheter was inserted into the portal vein and ligated The inferior caval vein was opened above and below the liver Injection of heparin (500 IE/100 g of body weight) was ... aggregat on

buffer containing 0 1 M sucrose as described previously (13) The liver tissue was cut into 1 mm thick cubes and slices and was fixed for 24 hours in the fixative mentioned above

Preparation for Microscopy

Tissues for light microscopy were embedded in paraffin and stained with hematoxylin-eosin and Perl s blue

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Note Figs 3–6 are electron micrographs of Kupffer cells from nonbled and bled animals Tissues fixed in glutaraldehyde thin sections stained with lead citrate Magnification $\times$ 30 000

Fig 3 Non bled one month after completed iron loading Numerous iron particles (IP) are present both in the cell sap and the lysosomes (Ly)

Fig 4 Bled one month after completed iron loading Lysosomes are seen to contain IPs However few IPs are seen in the cell sap Mi mitochondrion

Fig 5 Non bled two months after completed iron loading Large numbers of IPs both in lysosomes and cell sap

Fig 6 Bled two months after completed iron loading No IPs are present in the lysosomes or in the cell sap and the cell has the appearance of a normal rat liver Kupffer cell Ly lysosome Mi mitochondrion

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tions numerous iron positive granules were present in the liver parenchymal and Kupffer cells with the highest concentration in the periportal regions Within the parenchymal cells the iron positive granules were most abundant in peribilary regions

months showed a slight decrease in iron positive pigment in both parenchymal and Kupffer cells (Fig 1) The pigment was still most frequent in the periportal regions The pigment granules were also most concentrated in peribiliary areas of the parenchymal cells

Iron loaded bled animals Repeated phlebotomies did not result in a more rapid or extensive decrease in the total amount of iron positive pigment in the parenchymal cells than was observed in the non bled group The same localization with a higher concentration in the periportal regions was also noted However a drastic reduction in iron pigment was seen in the Kupffer cells and only small numbers of iron granules were seen after two months (Fig 2)

Electron Microscopy

Evidence of cell damage was not obtained in any of the cells studied at the fine structural level

Kupffer Cells

Iron loaded non bled animals Directly after completed

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an[illegible] [illegible] with tightly packed IPs type II bodies with a stretched-out tubular appearance also containing tightly packed IPs and bodies with a typical lysosomal structure All of these organelles were found to carry acid phosphatase and thus represented lysosomes (14) In the following they are referred to as lysosomes type I II and III In addition IPs were found diffusely dispersed in the cell sap

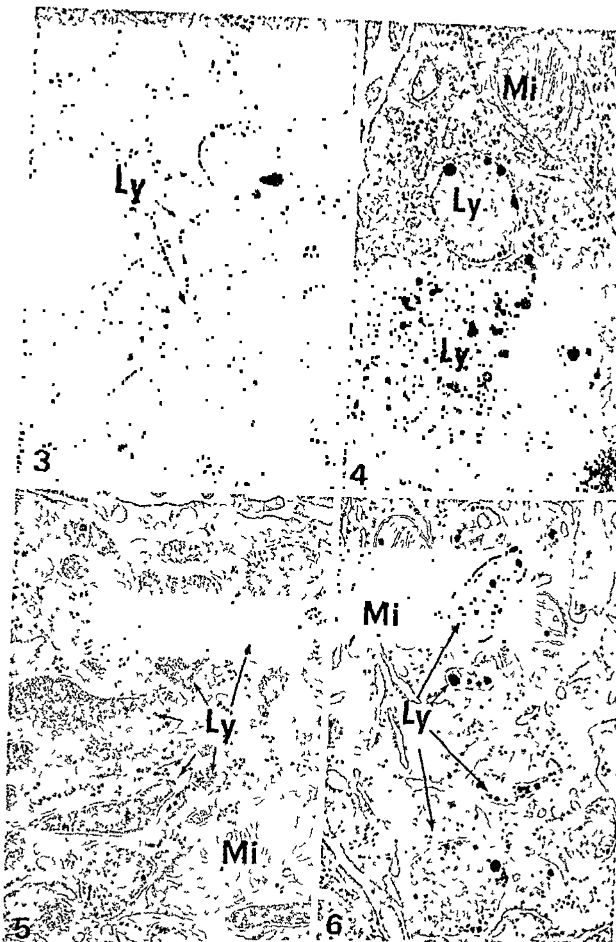
After one month IPs were present both in the cell sap and the three types of lysosomes mentioned above Type I lysosomes were rarely observed but when present all of them contained IPs though usually in lower concentration than after completed iron loading IPs were also observed in all type II and III lysosomes The number of IPs was generally lower than in corresponding elements directly after the loading Likewise the concentration of IPs in the cell sap was lower (Fig 3)

After two months IPs were seen in all type II bodies and conventional lysosomes [illegible]

roughly with the amount of intralysosomally located IPs being more frequent in the cells with a high concentration of IP in the lysosomes In the cells with a low concentration of IPs these particles were characteristically located in the periphery of the lysosomes IPs were not present in the nucleus mitochondria endoplasmic reticulum the Golgi apparatus or any other part of the cell

Iron loaded bled animals After one month the overall frequency of IPs had diminished considerably compared to the situation immediately after the Jectofer injection and the situation in the non bled group However some IPs were identified in the majority of the Kupffer cell lysosomes (Fig 4) The latter were usually of conventional type (type III) while type I and type II lysosomes were few or absent IPs were quite few in the cell sap and were not present in all cells

After two months no clearly identifiable IPs were seen within any membrane bound body or any part of the cell sap The lysosomal vacuome consisted mainly of two types of elements namely one with a rather dense matrix and another containing rounded structures with a globular appearance dispersed in a more translucent matrix Type I and type II lysosomes were not encountered (Fig 6) In general


Ly
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Ly
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Ne Figs 3–6 are electron micrographs of Kupffer cells from nonbled and bled animals Tissues fixed in glutaraldehyde thin sections stained with lead citrate Magnification" × 30 000

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tions numerous iron positive granules were present in the liver parenchymal and Kupffer cells with the highest concentration in the periportal regions Within the parenchymal cells the iron positive granules were most abundant in peribilary regions

Iron loaded non bled animals Compared to the liver tissue obtained immediately after iron loading the livers of the animals sacrificed after one and two months showed a slight decrease in iron positive pigment in both parenchymal and Kupffer cells (Fig 1) The pigment was still most frequent in the periportal regions The pigment granules were also most concentrated in peribiliary areas of the parenchymal cells

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Electron Microscopy

Evidence of cell damage was not obtained in any of the cells studied at the fine structural level

Kupffer Cells

Iron loaded non bled animals Directly after completed iron loading (13) IP s (electron dense iron containing particles) were present in three types of cytoplasmic bodies type I bodies which were large and rounded with tightly packed IPs type II bodies with a stretched-out tubular appearance also containing tightly packed IPs and bodies with a typical lysosomal structure All of these organelles were found to carry acid phosphatase and thus represented lysosomes (14) In the following they are referred to as lysosomes type I II and III In addition IPs were found diffusely dispersed in the cell sap

After one month IPs were present both in the cell sap and the three types of lysosomes mentioned above Type I lysosomes were rarely observed but when present all of them contained IPs though usually in lower concentration than after completed iron loading IPs were also observed in all type II and III lysosomes The number of IPs was generally lower than in corresponding elements directly after the loading Likewise the concentration of IPs in the cell sap was lower (Fig 3)

After two months IPs were seen in all type II bodies and conventional lysosomes and also in the cell sap of all Kupffer cells (Fig 5) The concentration of IPs in the cell sap and within limited bodies varied considerably between different cells Characteristic type I bodies were lacking in these cells The concentration of IPs in the cell sap correlated roughly with the amount of intralysosomally located IP s being more frequent in the cells with a high concentration of IP in the lysosomes In the cells with a low concentration of IPs these particles were characteristically located in the periphery of the lysosomes IPs were not present in the nucleus mitochondria endoplasmic reticulum the Golgi apparatus or any other part of the cell

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Ly

Mi

Ly

Ly

3

4

Mi

Ly

Ly

Mi

5

6

Fig 7 Peribiliary region of two rat liver cells from a non bled animal two months after completed iron loading The lysosomes are packed with IPs IPs are also frequent in the cell sap Bc bile canaliculus ER endoplasmic reticulum Go Golgi apparatus Ly lysosome Mi mitochondria P peroxisomes Glutaraldehyde lead citrate × 25 000

Inset IPs are tightly packed in a lysosome but more sparse in the surrounding cell sap × 40 000

Fig 8 The appearance of peribiliary regions of three adjacent liver cells from a bled rat (2 months) is illustrated Numerous IPs are seen both in lysosomes (Ly) and the cell sap Bc bile canaliculi Gly glycogen Go Golgi apparatus Mi mitochondria Glutaraldehyde Staining with lead citrate × 30 000

Inset (a) shows a lysosome filled with IPs IPs are also scattered in the surrounding cell sap Inset (b) shows an apparent fusion between an IP-containing lysosome and an autophagic vacuole (AV) × 40 000

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the Kupffer cells and their lysosomal system showed the appearance previously described in the normal rat (27)

Parenchymal Cells

Iron loaded non bled animals Directly after completed iron loading there are three types of IPcontaining bodies present in the parenchymal cells one with a typical lysosome like appearance (peribiliary dense body) one containing no other visible material than IPs and a third body with the appearance of autophagic vacuoles All of these organelles have been shown to contain acid phosphatase and hence represent lysosomes (14) Great numbers of IPs were observed in the cell sap

After one month all parenchymal cells showed presence of IPs in the cell sap however not as concentrated as immediately after loading (13) Completely IP filled lysosomes lacking other materials than the granules were no longer present IPs were usually although not always observed in the other two types of lysosomes described above Lysosomes lacking IPs were characterized by occurrence of abundant membranous and dense materials and appeared to correspond to residual bodies In many cases the IP-carrying lysosomes also contained other (degraded) materials

After two months some IPs were found in all parenchymal cells The concentration of IPs in the cell sap was not clearly lower than at the one month intervals IPs were also located in the lysosomes (of conventional type and in the form of autophagic vacuoles) In the latter the IPs [illegible]

vacuoles (Fig 7) with material such as glycogen and IP separated inside the vacuole There was no clear difference concerning the concentration of IPs between lysosomes in cells at the one and two month intervals IPs were not demonstrated in any other cellular organelle or compartment than those mentioned above

Iron loaded bled animals After one month IPs were seen in all parenchymal cells in the liver lobule in both the cell sap and different types of lysosomes No clear difference in IP-content in the lysosomes or the cell sap could be demonstrated between this group and the non bled group

After two months considerable numbers of diffusely distributed IPs were still present in the cell sap IPs were also observed in membranelimited bodies but only two varieties namely autophagic vacuoles and lysosomes of the peribiliary dense body type (completely IP filled lysosomes were no longer present) (Fig 8) The IPs were sparse in some lysosomes of the lysosomes of the residual body type and also in large irregular bodies containing globular and osmiophilic material and probably representing lysosomes transforming into residual bodies However in most lysosomes IPs were rather abundant Sometimes a compartmentalization occurred in the autophagic vacuoles resulting in a separation of the IPs from concomitantly present autophagocytosed matter There were no signs of fusion between IP-containing bodies and the plasma membranes However images indicating fusion between IP-containing secondary lysosomes and autophagic vacuoles were seen suggesting that the digestive function of secondary lysosomes was not impaired by the presence of iron loading (Fig 8) No clear difference in the frequency of IPs between the bled and the non bled group was noted in this cell type This applies both for the cell sap and the lysosomes No IPs were identified in other fractions than the cell sap or the lysosomal vacuome Neither was IPs seen outside the cell in the bile capillaries of the space of Disse

Endothelial and Fat Storing Cells

Like immediately after iron loading no IPs could be discovered in any part of these cells The same applies for both the bled and non bled animals as well as those in the control groups

P
Mi
Ly
Ly
Go
Ly
ER
Bc
7
a
Ly
Bc
Gly
Mi
Ly
Ly
Go
Ly
Av
8

Figs 9–10 Parts of Kupffer cells from a rat which had been given iron two months previously The cell in *Figure 9* is from a non bled animal and in *Figure 10* from a bled animal The tissues have been incubated for the demonstration of acid phosphatase The granular nature of the reaction product in lysosomes makes it hard to detect possible dual occurrence of IPs Ly lysosome Nu nucleus Glutaraldehyde incubation time 45 min section staining with lead citrate × 21 000

Fig 11 Liver parenchymal cell from an unbled rat (2 months after completed iron loading) Tissue incubated for the demonstration of acid phosphatase containing several bodies presumed to represent lysosomes One apparent lysosome (Ly_1) is packed with IPs but does not show clear evidence of final product Two autophagic vacuoles (AV) contain both reaction product and IPs (arrows) while a lysosome (Ly_2) clearly is covered by lead phosphate precipitate and may contain IPs (these seem to be obscured by the precipitate) Mi mitochondria Glutaraldehyde incubation time 45 min section staining with lead citrate × 38 000

Note Figs 12 14 show a series of pictures from tissue incubated for the demonstration of acid phosphatase showing the postulated development of an autolysosome through fusion of an autophagosome with a primary lysosome Glutaraldehyde incubation time 45 min section staining with lead citrate × 50 000

Fig 12 An autophagic vacuole (AV) containing undegraded glycogen and IPs The vacuole lacks evidence of reaction for phosphatase

Fig 13 An autophagic vacuole (AV) containing undegraded glycogen and IPs but no reaction product Adjacent to vacuole is a smaller structure bel eved to be the equivalence of a primary lysosome (PL) with deposits of lead sphate precipitate This lysosome is believed to be in the precess of fusing with the autophagic vacuole

14 A lysosome (Ly) showing presence of reaction product and IPs

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HISTOCHEMISTRY

ffer Cells

Incubation for the demonstration of acid phosatase resulted in deposition of electron dense ction product over lysosomal structures often king the concomitant occurrence of IPs and ction product hard to substantiate This applies most of the IP-containing lysosomes

Iron loaded non bled an mals Reaction product is encountered in all membrane bound bodies here IPs were identified both after one and two onths (Fig 9) However it was sometimes fficult to identify the IPs in the individual bodies ie to the simultaneous presence of reaction oduct in the lysosomes Lead phosphate deposits ere also observed in the Golgi apparatus

Iron loaded bled animals Distinct deposits of action product were identified over the different pes of lysosomal vacuoles both after one and two onths Also in these groups reaction product was en in the Golgi apparatus Other portions of the lls lacked precipitates At the one month interval ome reaction product-containing bodies showed resence of IPs while IPs appeared to be lacking in uch bodies two months after completed iron oading

Parenchymal Cells

Iron loaded non bled animals Reaction product was deposited over lysosomal elements in the parenchymal cells Some of these bodies showed a diffuse heavy deposition of precipitate – in many instances rendering it difficult or impossible to identify concomitantly occurring IPs Some IP containing autophagic vacuoles lacked reaction product (Fig 12) Images suggesting fusion between lysosomes and such IP-containing autophagic vacu oles were also obtained (Fig 13 and 14) Deposition of final reaction product was however observed over other autophagic vacuoles and also in portions of the Golgi apparatus

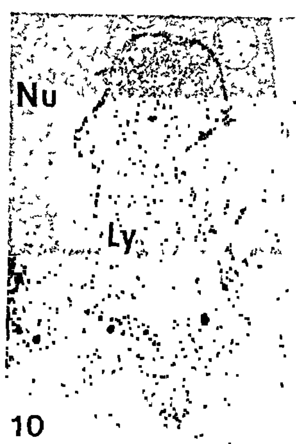
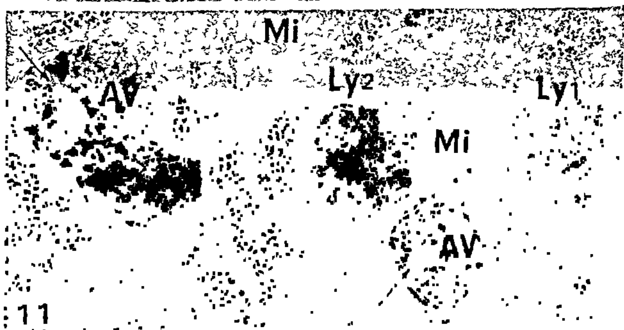
Iron loaded bled animals The lysosomal vacu ome showed presence of deposits of reaction product in bodies also containing IPs However reaction product was also noted in structures where the distinct features of IPs could not be revealed These structures usually corresponded to somewhat enlarged peribiliary dense bodies lacking recogni zable content or sign of autophagy some of them bore resemblance to residual bodies Reaction product was further deposited over many (but not all) autophagic vacuoles some of which encompas sed IPs Some portions of the Golgi apparatus showed presence of reaction product

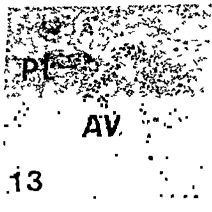
Endothelial and Fat Storing Cells

Lysosomes in endothelial cells of both groups showed a distinct deposition of reaction product The occasionally encountered fat storing cells also contained bodies covered by precipitates

Controls

No reaction product was demonstrated in any of the incubated controls


Ly
Ly
9

Nu
Ly
10

Mi
AV
Ly2
Ly1
Mi
AV
11

AV
12

Pt
AV
13

Ly
14

gs 9–10 Parts of Kupffer cells from a rat which had been given iron two months previously The cell in *Figure 9* is om a non bled animal and in *Figure 10* from a bled animal The tissues have been incubated for the demonstration acid phosphatase The granular nature of the reaction product in lysosomes makes it hard to detect possible dual currence of IPs Ly lysosome Nu nucleus Glutaraldehyde incubation time 45 min section staining with lead trate ×21 000

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Fig 14 A lysosome (Ly) showing presence of reaction product and IPs

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HISTOCHEMISTRY

Kupffer Cells

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Iron loaded non bled animals Reaction product was encountered in all membrane bound bodies where IPs were identified both after one and two months (Fig 9) However it was sometimes difficult to identify the IPs in the individual bodies due to the simultaneous presence of reaction product in the lysosomes Lead phosphate deposits were also observed in the Golgi apparatus

Iron loaded bled animals Distinct deposits of reaction product were identified over the different types of lysosomal vacuoles both after one and two months Also in these groups reaction product was seen in the Golgi apparatus Other portions of the cells lacked precipitates At the one month interval some reaction product-containing bodies showed presence of IPs while IPs appeared to be lacking in such bodies two months after completed iron loading

Parenchymal Cells

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Endothelial and Fat Storing Cells

Lysosomes in endothelial cells of both groups showed a distinct deposition of reaction product The occasionally encountered fat storing cells also contained bodies covered by precipitates

Controls

No reaction product was demonstrated in any of the incubated controls

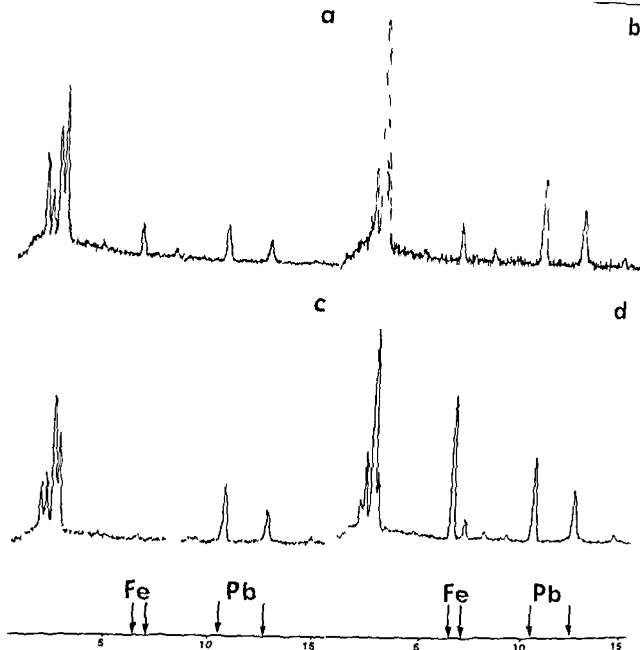


Fig 15 Energy spectrum obtained from Kupffer and parenchymal cells A and B show spectra from iron loaded non bled Kupffer (a) and parenchymal cell (b) lysosomes C and D show spectra from Kupffer and parenchymal cell lysosomes of bled animal respectively Note the absence of iron in the Kupffer cell lysosome from the bled animal Energy quanta in KeV at which the signals for different elements were recorded Fe (iron) Kα 6 4 Kβ 7 1 Pb (lead) Mα2 4 Lα 10 5 Lβ 12 6 Cl (chlorine) Kα 2 5 and P (phosphorous) Kα 2 0

X RAY MICROANALYSIS

The graphs recorded from the different cell types are shown in Figure 15

The same outcome in the analysis for presence of iron was obtained before and after incubation for the demonstration of acid phosphatase in all instances the following description will therefore only deal with the incubated material

Kupffer Cells

Iron loaded non-bled animals In this group all types of lysosomes showed presence of both iron and lead (reaction product) after one and two months Peaks for lead only were obtained from the Golgi regions Small peaks indicating presence of iron were obtained from the cytoplasmic ground substance Signals for iron or lead were not received from any other part of the cells

Iron loaded bled animals All bodies containing iron gave a positive peak for lead After two months peaks for iron were not recorded in any Kupffer cell whereas peaks for lead from lysosome like structures were received in the same way as from Kupffer cells of the other groups Iron and lead were not demonstrated in any other part of the cell Signals indicating presence of lead only were obtained from the Golgi regions

Parenchymal Cells

Iron loaded bled and non bled animals No difference in the distribution of iron or lead has been seen in this cell type between the different groups After one month the peaks for iron were similar to those received immediately after iron loading After two months some peribiliary dense bodies showed a high concentration of iron (Fig 15) compared to the bodies investigated previously Presence of iron was also recorded in the graphs from the cell sap Signals for lead were also recorded from some Golgi regions but not from any other parts of the cells

Endothelial Cells and Fat Storing Cells

Only lead – and no iron – was found in the lysosomes analysed in these cell types No significant signals for lead or iron was received from any other part of the cell

Controls

No peaks for iron were recorded in the animals *only* given saline Peaks for lead were obtained from typical lysosome like structures in all cells

No peaks for lead were seen in the tissues from the experimental animals used for histochemical controls while the peaks for iron were the same as in liver tissue incubated in the proper medium for the demonstration of acid phosphatase

DISCUSSION

The present work demonstrates that during the mobilization of iron from the iron loaded rat liver by way of bleeding Kupffer cells show a rapid decrease in and almost complete disappearance of IPs while there is only a limited decline in the IPs present in the parenchymal cells Depletion of IPs in the latter cells was not noted despite extensive bleeding of the animals It was further observed that all IP-containing cytoplasmic bodies also showed presence of acid phosphatase one and two months after cessation of iron loading implying that all of these bodies represent lysosomes

Previous studies by *Shoden and Sturgeon* (25) concerning the mobilization of iron from the liver following overload demonstrated – on the light microscopical level – that Kupffer cell hemosiderin was more readily removed than parenchymal cell hemosiderin after administration of saccharated iron oxide followed by bleeding It has further been shown (9) that iron taken up by »reticuloendothelial (Kupffer) cells in the liver is released in an early and a late phase However if body iron was reduced by phlebotomy the Kupffer cells were able to release most of the excess iron in the early phase This means that they have the ability to alter their release of iron when there is a need for it It has likewise been shown (3) that release of iron from reticuloendothelial cells is increased in humans with iron deficiency However nothing was revealed

when the demand for iron was increased the stored iron was readily released from the Kupffer cells while the endothelially stored iron was retained *Hershko et al* (11) among others has performed studies on the effects of a chelating agent – desferrioxamine – and demonstrated that administration of this substance mainly results in reduction of the iron overload in parenchymal cells *Bradford et al* (5) have claimed that ferritin and hemosiderin are released into the bile through reversed pinocytosis by parenchymal cell lysosomes after loading with iron

In previous papers we have discussed the possible ways of

22) and – following binding of iron – becomes sequestrated in lysosomes by way of autophagy as suggested by *Trump et al* (26) among others In the present study we noted that IPs were present in early autophagic vacuoles along with undegraded glycogen IPs were demonstrated also in an autophagic vacuole lacking reaction product which appeared to fuse with a structure with heavy deposition of final product. This observation seems to support the idea that IPs are incorporated together with other material from the cell sap in the lysosomal vacuome The morphological and cytochemical observations have not indicated other ways by which IP s might become associated with the interior of lysosomes It appears unlikely that iron could become bound to apoferritin in lysosomes since apoferritin is rapidly degraded

their iron moiety to the blood to become bound in transferrin when needed (9) However it has also been suggested that ferritin molecules in the cell sap or in membrane bound vacuoles of different cells might be released directly into the blood stream since the serum level of this protein is increased in different iron storage diseases (21) Such release is evidently very difficult to demonstrate morphologically However in our pictures we never encountered any evidence for a release of this kind Furthermore other macromolecules – such as thorium dioxide particles – that are not degraded by lysosomal enzymes have been shown to remain inside lysosomes of Kupffer cells for up to a year (8 28) This militates against the idea that reversed endocytosis of lysosomal content had occurred in the Kupffer cells The occurrence in the peripheral blood of ferritin molecules in various iron storage diseases (21) may possibly be explained by reversed endocytosis in other types of cells Alternatively bits of cytoplasm with ferritin in the cell sap or vacuolar elements might become dispatched into the blood stream whereby the ferritin molecules could be set free through lysis of the cytoplasmic fragment

If not complete ferritin molecules are released from the cells disappearance of IPs could be explained by separation of the iron moiety from the ferritin molecule One could then hypothesize the cell sap ferritin is first depleted of its iron molecules and that the lysosomally stored ferritin and hemosiderin molecules subsequently release their iron and become degraded Another possible way is that the cell sap ferritin is first autophagocytosed in order to be intralysosomally degraded for subsequent release of iron

The impression obtained from the pictures was that in Kupffer cells of the bled animals IPs in the cell sap decreased before those in the lysosomes suggesting that the release of iron commences from the cell sap when the release is increased However the evidence obtained did not reveal whether or not this postulated transport occurred through movement of ferritin molecules across the cell membrane or in some other way (such as transfer of iron atoms)

The possible ways for iron transport from the parenchymal cells are (a) release of ferritin molecules of the iron moiety to the blood at the sinusoids and (b) transport to the bile As regards the release of iron for binding to transferrin in the blood the same ways are possible as indicated concerning the Kupffer cells Presence of iron and ferritin molecules in the bile has been demonstrated by Bradford et al (5) Theoretically the transport of iron to the bile can be viewed to occur in at least 3 different ways One might be through release of free iron atoms from the cell sap to the bile canaliculus a second transmembrane movement of ferritin molecules residing in the cell sap and a third reversed endocytosis from lysosomes as suggested by Bradford et al (5)

We have not noticed any signs of reversed endocytosis in the peribilliary regions or elsewhere and we have therefore no evidence to support this theory The ferritin molecule is too large to be able to be transported through the plasma membrane Alternatively transport of iron atoms across the cell membrane into the bile might occur It should be emphasized however that liver parenchymal cells are said to be able to excrete lysosomal content by reversed endocytosis when being under the influence of toxic substances such as arabitosin and vinblastine (15 18)

The difference in IP content between Kupffer cells in the bled and non bled groups is striking The fact that the Kupffer cells lose IPs more rapidly than the parenchymal cells in the bled group can be explained in several ways One possibility is of course that the parenchymal cells have larger stores if the rate of release is the same from both cell types the Kupffer cell will evidently by depleted before loss from the parenchymal cell is substantial This does not seem likely since in the normal rat the Kupffer cells have a volume density of lysosomes that is approximately 50% of that in parenchymal cells (4) furthermore it appears that the Kupffer cell lysosomes are the ones that increase in volume the most after iron overload indicating that they initially might store a large portion of the IPs as was pointed out previously (13) The other possibility would be that the Kupffer cells release iron more readily than the parenchymal cells our present findings are compatible with this hypothesis One possibility is of course that the initially stored Kupffer cell iron becomes redistributed to both parenchymal and hemopoietic cells resulting in a depletion of iron in Kupffer cells and a continuing overload in the parenchymal cells In order to prove this redistribution model one would have to isolate parenchymal and sinusoidal cells after different periods and measure the iron content in both cell types separately

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COMPARATIVE SINGLE CELL AND FLOW DNA ANALYSIS IN ASPIRATION BIOPSIES FROM BREAST CARCINOMAS

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Auer G & Tribukait B Comparative single cell and flow DNA analysis in aspiration biopsies from breast carcinomas Acta path microbiol scand Sect A 88 355–358 1980

The DNA distribution patterns in fine needle aspirates from 17 breast carcinomas were analysed using single cell and flow cytophotometric techniques A good correlation was observed to exist between the modal DNA values obtained by the two methods Advantages and disadvantages of the two methods are discussed

Key words DNA analyses human breast carcinoma

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Fine needle aspiration biopsy is increasingly used for diagnosis of palpable breast lesions The main advantage of this technique is its high diagnostic accurancy combined with speed and patient acceptability Since fine needle biopsy is minimal traumatic it also allows repeated sampling for example during cytostatic or endocrine treatment of inoperable tumors

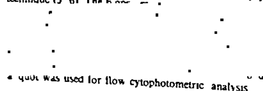
Recent studies have shown that DNA measurements in single morphologically identified breast cancer cells from fine needle aspirates are valuable for the prediction of patient survival (1) In addition a strong correlation between DNA distribution pattern and estrogen receptor (ER) content in breast cancer has been demonstrated indicating that DNA measurements may be used in order to estimate the sensitivity of breast cancer to endocrine therapy in cases in which ER levels cannot be determined with sufficient accurancy i e in premenopausal women (2) It is thus clear that DNA measurements in fine needle aspirates contribute valuable information concerning prognosis and choice of therapy of breast cancer patients

In the present investigation we studied whether the relatively time-consuming single cell DNA measurements in breast cancer aspirates can be replaced by flow DNA measurements which would decisively facilitate the use of DNA analysis in routine patient work

MATERIAL AND METHODS

Cell Material

Cell samples from human breast carcinoma were obtained by means of fine needle aspiration biopsy technique (5 6) The h



quot was used for flow cytophotometric analysis

Fine Needle Aspiration Biopsy

The technique consists of inserting a thin (0 5–0 7 mm o d) needle into a palpable mass e g in the present investigation into a breast tumor and aspirating cellular

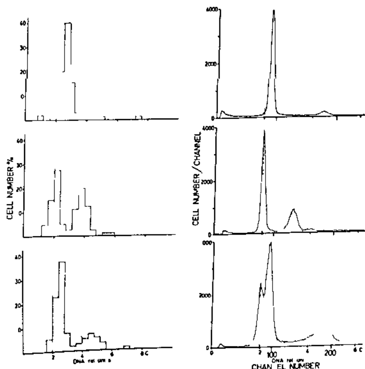
material into the lumen of the needle by negative pressure produced by drawing back the plunger of a 10 ml syringe attached to the needle During sampling the needle is moved back and forth several times through the tumor mass usually at different angles to the original angle of insertion The aspirated cell material consists of single cells or clumps of cells In breast cancer generally the vast majority of the aspirated cells were found to be tumor cells Small amounts of non tumor cells especi ally leucocytes benign mammary epithelial cells fibrob lasts and fat cells are usually admixed with the tumor cells

Single Cell Cytophotometry

Smear preparations were fixed in buffered 4% formaldehyde for a minimum of 15 hours and Feulgen stained Previous studies (7) showed that hydrolysis for 1 hour at 22 °C in 5 M HCL resulted in optimal staining intensities The nuclear Feulgen DNA in single cells was measured in a rapid scanning – integrating microspectro photometer developed by *T Caspersson* (3 4) Diploid amounts of DNA were determined by using leucocytes and normal mammary epithelium adventitiously present in all aspirates as internal control The discrepancies between DNA values of leucocytes with condensed chromatin were partially corrected for by measuring at the »off peak« wave length of 610 nm Usually 100 tumor cells and 30 control cells were measured per each preparation Tumor cells could easily be distinguished from normal cells either by their nuclear morphology or by the additional use of phase contrast All Feulgen DNA values were expressed in relation to their corresponding staining control which was given the value 2 c denoting the normal diploid DNA content

Flow Cytophotometry

Flow cytophotometric measurements were performed according to a previously described procedure (8) The cell material from fine needle aspirates was fixed in ice cold ethanol after washing in buffer solution (0 1 M Tris 0 07 M NaCl 0 005 M EDTA pH 7 5) After at least 12 hours fixation the cell material was washed in buffer solution with 1 mg of RNase per ml (RNase A Sigma) added Single cell nuclei were obtained by subsequent treatment with pepsine (0 5% pepsine Merck No 7180 1000 Eg) for 10 minutes at 37 °C After further washing the DNA in the cell nuclei was stained with ethidium bromide (2 5 × 10^{-5}M Serva Heidelberg Nr 21238 in Tris EDTA buffer 386 mOsm) All prepara tions were checked microscopically The DNA contents of the cell nuclei were analysed using a rapid flow cytofluorometer ICP 11 (Phywe W Germany) with a flow rate of up to 1000 cells/sec The excitation and emission wavelengths were 455–490 nm and 590–630 nm The output was sorted by a 256 channel analyser (Nuclear Data 100) and was presented as a histogram The DNA values were expressed in relation to the DNA content of normal diploid human cells Calculation of the proportion of G_1 S and G_2 + M cells was achieved by automatic integration of cells into corresponding chan nels in the multichannel analyser The values were corrected for background noise On average 30 000–50 000 cells per aspirate were measured



RESULTS

Fig 1 illustrates three examples of DNA-distribution patterns of breast carcinomas obtained by both single cell and flow cytophotometric technique It can be seen from the figure that the corresponding DNA-profiles of the first and the second tumor (Fig 1 upper and intermediate curves) are more or less the same Thus in both the single cell and flow cytophotometric measurements the first tumor exhibits a distinct modal DNA value in the hyperdiploid region of normal mammary epithelial cells and the second tumor exhibits distinct modal DNA values in the diploid and hypotetraploid regions In the third tumor (Fig 1 lower curves) the single cell DNA profile shows modal DNA values in the hyperdiploid and hypertetraploid regions whereas the flow DNA profile shows DNA values in the diploid hyperdiploid and hypertetraploid region indicating the presence of a large number of diploid normal cells in the aspirate

Fig 2 illustrates the correlation between modal DNA values of 17 breast carcinomas obtained by single cell and flow cytophotometric measurements Since flow cytophotometry does not allow discrimination between normal mammary epithelial cells and tumor cells containing DNA amounts corresponding the normal diploid amount only modal DNA values below or above the normal 2 c value were compared It is clear from the figure that in aspirates from breast carcinomas a good correlation exists between modal DNA values obtained by means of single cell cytophotometric measurements and those obtained by means of flowcytophotometric measurements

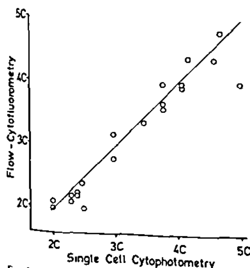


Fig 2 Relationship between model DNA values (with the exception of those exhibiting values at 2 c of normal cells) obtained by using single cell and flow cytophotometry The results comprise measurement in aspirates from 17 breast carcinomas in 3 cases 2 distinct model DNA values were present

DISCUSSION

Single cell DNA cytophotometry in fine needle aspirates from breast carcinomas has been demonstrated to be valuable for the prediction of the clinical course in the individual patient over and above that reflected by clinical stage and morphological criteria Patients with tumors in which nearly all cells exhibited DNA values in the near-diploid region were found to have a favourable prognosis whereas in patients with increased and scattered DNA values the prognosis was poor (1)

In the present work we investigated whether single cell DNA analysis in breast cancer aspirates could be replaced by flow DNA analysis Cytophotometric single cell DNA analysis is still a relatively time-consuming procedure and therefore scarcely suitable in routine clinical work Flow cytophotometry on the other hand permits rapid DNA measurements of large cell numbers within a minimal time period

Our results show that the DNA profiles obtained by the two methods are well correlated to each other It is thus clear that in aspirates from breast tumors single cell DNA measurements can be replaced by flow DNA measurements Besides rapidity the advantage of the flow technique is the high degree of resolution due to the large number of cells measured Fine needle aspiration material was found to be espicially suitable for flow measurements since the samples are made up of single cells and small cell complexes which means that well preserved cell nuclei can more easily be prepared in comparison with surgical biopsy material Furthermore fine needle aspirates contain in general mainly tumor cells which minimizes possible alteration of flow DNA profiles due to excessive admixture of normal cells Yet since breast carcinomas may be composed of tumor cells with DNA amounts corresponding with that found in normal cells e g tumor cells with DNA-contents in the normal diploid region the cellular composition of each sample has to be investigated cytomorphologically in order to be able to interpret flow DNA measurements

Supported by *Stockholms Cancer Society* and the *Swedish Cancer Society*

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Acta path microbiol scand Sect A 88 359–367 1980

BRONCHIOLAR EPITHELIAL LESIONS INDUCED IN THE PREMATURE RABBIT NEONATE BY SHORT PERIODS OF ARTIFICIAL VENTILATION

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Nilsson R Grossmann G & Robertson B Bronchiolar epithelial lesions induced in the premature rabbit neonate by short periods of artificial ventilation Acta path microbiol scand Sect A 88 359–367 1980

Premature newborn rabbits were ventilated with standardized tidal volume (10 ml/kg) or standardized insufflation pressure (35 cm H_2O) for 1–30 min The lungs of the experimental fetuses were fixed by perfusion of the pulmonary artery and examined by light and electron microscopy with particular reference to the prevalence and ultrastructure of bronchiolar epithelial lesions Necrosis and desquamation of bronchiolar epithelium were constant findings in fetuses ventilated for 5 min or more and were also present in some of the fetuses ventilated for only 1 min Since the light microscopic and ultrastructural appearance of these bronchiolar lesions is similar to early stages of human neonatal hyaline membrane disease we conclude that artificially ventilated pre term newborn rabbits might serve as a useful model of this disease

Key words Artificial ventilation bronchiolar epithelial lesions hyaline membrane disease IRDS premature newborn rabbit.

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Accepted as submitted 29 v 80

Necrosis and desquamation of bronchiolar epithelium are regular findings early in the course of the neonatal »idiopathic respiratory distress syndrome« (IRDS) (*Gandy et al* 1970 *Finlay Jones et al* 1974) and the same type of lesion has been observed in pre term newborn experimental animals subjected to artificial ventilation (*Stahlman et al* 1964 *McAdams et al* 1973 *Schwieler and Robertson* 1976 *Adams et al* 1978 *Cutz et al* 1978 *Nilsson et al* 1978 *Nilsson* 1979) It therefore seems likely that when infants suffering from severe IRDS are treated with positive pressure ventilation there is a risk that the respirator treatment will aggravate the »endogenous« epithelial lesions This might in turn lead to the complication known as »broncho pulmonary dysplasia« – a syndrome of chronic pulmonary sequelae which is seen particularly in IRDS patients in whom high peak pressures have been used during the period of artificial ventilation (*Taghizadeh and Reynolds* 1976)

Since most previous experimental studies in this field were carried out without adequate standardization of the ventilator treatment there is no precise information about the pathogenesis and early development of the bronchiolar epithelial lesions We therefore initiated a series of experiments in which premature newborn rabbits were ventilated with a standardized tidal volume (V_T) for defined time periods and the extent of epithelial lesions was assessed by histologic morphometric techniques In addition the fine structure of the early epithelial lesions was examined by electron microscopy

MATERIAL AND METHODS

The experiments were carried out on 44 premature rabbit neonates delivered on day 27 of gestation (full term = 31 ± 1 day) (Table 1) The fetuses were obtained by

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8. *Tribukait B, Gustafson, H & Esposti P* Ploidy and proliferation in human bladder tumors as measured by flow cytofluorometric DNA analysis and its relations to histopathology and cytology Cancer *43* 1742–1751, 1979

.E 2 *Lung Thorax Compliance and Insufflation Pressure in Premature Rabbit Neonates Delivered on Day 27 of Gestation*

e interval (min)	Number of observations	Compliance (ml/cm H_2O kg)	P vs preceding time interval	Insufflation pressure (cm H_2O)	P vs preceding time interval
0–1	20	0 26 ± 0 03		38 ± 3	
2–5	14	0 28 ± 0 05	N S	35 ± 5	N S
6–10	10	0 28 ± 0 05	N S	37 ± 5	N S
26–30	5	0 31 ± 0 05*	N S	33 ± 5	N S

vs time interval 0–1 min <0 02

recordings were obtained during intermittent positive pressure ventilation with standardized tidal volume (10 ml/

Values are given as $\bar{X} \pm SD$ for various time intervals after the onset of ventilation

RESULTS

g Thorax Compliance

n animals ventilated for more than 1 min , the ssure needed to maintain the standardized V_T reased slightly during the period of observation other words there was a gradual increase in the npliance of the lung thorax system However, a tistically significant difference was found only ween the intervals 0–1 min and 26–30 min ble 2)

Lung thorax compliance for animals used for ctron microscopy was 0 25 ± 0 07 ml/cm O kg ($\bar{X}$ ± SD) after 5 min of artificial ntilation this value is very close to the compliance ure for the corresponding interval in Table 2

stologic Morphometric Findings

In all fetuses there was poor aeration of the veolar compartment as verified by low figures for and there was no difference between fetuses ventilated for various periods of time (Table 3) Epithelial lesions were absent in all non-ventilated fetuses However, necrosis and desquamation of the bronchiolar epithelium was a constant finding in all fetuses which had been ventilated for 5 min or more In the group ventilated for only 1 min , pyknotic bronchiolar epithelial cells were found in 2 of 7 fetuses (Table 3 Fig 1A)

of the individual lesion changed however through out the period of observation Desquamated cells accumulated in the peripheral airways in later intervals and hyaline membranes made up of cell debris from necrotic epithelium gradually became more prominent (Fig 1A–C) Figures for I_b representing various periods of artificial ventilation are shown in Table 3

ABLE 3 *Morphometric Data on Alveolar Expansion and Bronchiolar Epithelial Lesions in Premature Rabbit Neonates Delivered on Day 27 of Gestation Related to the Duration of Artificial Ventilation*

uration of entilation min)	n	I_a	Bronchiolar epithelial lesions			
			Number of fetuses		I_b	P vs preceding time interval
			with lesions	without lesions		
0	11	0 71 ± 0 25	0	11	0	
1	7	0 79 ± 0 29	2	5	0 003 ± 0 005	N S
5	6	0 61 ± 0 05	6	0	0 16 ± 0 03	= 0 002
10	5	0 82 ± 0 26	5	0	0 29 ± 0 11	= 0 05
30	5	0 76 ± 0 35	5	0	0 27 ± 0 09	N S

Values are given as $\bar{X} \pm SD$

Table 1 *Survey of the Material*

Period of artificial ventilation (min)	Parameters evaluated			
	Compliance + $I_a + I_b$		Ultrastructure of bronchiolar epithelium	
	n	BW (g $\bar{X} \pm$ SD)	n	BW (g $\bar{X} \pm$ SD)
0	11	30 ± 7	5	27 ± 7
1	7	31 ± 3		
5	6	30 ± 2	5	23 ± 4
10	5	25 ± 5		
30	5	27 ± 6		

BW = body weight

hysterotomy under intravenous anesthesia (Ketalar® Parke Davis MI USA) or following sacrifice of the doe by rapid injection of 2 ml sodium pentobarbital (Mebumal vet® 60 mg/ml ACO Sweden) and 5 ml potassium chloride (150 mg/ml) Non ventilated fetuses were killed *in utero* with an intraperitoneal injection of 0 5 ml Mebumal vet®

Artificial Ventilation

Immediately after delivery the fetuses received intra peritoneal injections of 0 1 ml sodium pentobarbital (6 mg/ml) and 0 1 ml pancuronium bromide (Pavulon® 0 2 mg/ml Organon Holland) for anesthesia and muscle relaxation They were tracheotomized kept at 37 °C in a volume constant body plethysmograph and connected to a Harvard Rodent Ventilator 680 set at a frequency of 60/min In the incubator the fetuses were fixed in dorsal position by ECG electrodes through their paws Fetuses were included in the study only if the ECG recording revealed cardiac activity throughout the period of ventilation

Insufflation pressure and pressure in the plethysmo graph were registered simultaneously by pressure trans ducers (EMT 33 or 34 Siemens Elema Solna Sweden or Statham Statham laboratories USA) and recorded on a Mingograph 24B or 81 (Siemens Elema) or on a Polygraph 7B (Grass Incorp USA) Fetuses used for determination of lung thorax compliance and morpho metry were ventilated with air with standardized V_T (10 ml/kg) for periods varying between 1 and 30 min The quasistatic compliance of the lung thorax system was calculated from registrations of V_T and insufflation pressure at end inspiration

Histologic and Morphometric Techniques

After artificial ventilation the fetuses were killed with intraperitoneal sodium pentobarbital (0 5 ml 60 mg/ml) The lungs were fixed by perfusion of the pulmonary artery with 1 per cent glutaraldehyde and 3 5 per cent formaldehyde in phosphate buffer at a pressure of 65 cm H_2O During the perfusion procedure (15 min) the air expansion of the lungs was maintained by an endotra cheal pressure of 15 cm H_2O The whole lungs were then embedded in paraffin and sections from the basal portions were stained with hematoxylin and eosin and examined microscopically By means of the point counting technique (*Chalkley* 1943) we determined the relative volumes of the alveolar spaces (V_a) and solid parenchyma (V_p) and the »alveolar expansion index« (I_a) was calculated by the formula (*Enhorning et al* 1973)

$$I_a = \frac{V_a}{V_p}$$

The same sections were then re-examined and the number of high power fields (HPFs) showing evidence of bronchiolar epithelial necrosis were counted and related to the total number of HPFs examined The »index of bronchiolar epithelial lesions« (I_b) was calculated according to the formula (*Nilsson et al* 1978)

$$I_b = \frac{\text{No of HPFs with lesion}}{\text{No of HPFs examined}} \times I_a$$

In this calculation I_a is used as a correction factor to compensate for variations in the volume of the alveolar compartment that might influence the spacing of the bronchioles

To avoid bias the histologic morphometric evaluation of the sections was made without knowledge of the experimental conditions to which the individual fetuses were subjected

Electron Microscopy

This part of the study was carried out on a separate series of 5 fetuses ventilated for 5 min with a standardized insufflation pressure of 35 cm H_2O and a V_T of 9 0 ± 2 8 ml/kg ($\bar{X}$ ± SD) Non ventilated fetuses were used as controls (Table 1)

For transmission electron microscopy (TEM) speci mens representing marginal parts of the lungs were embedded in Vestopal following dehydration in acetone Semithin and ultrathin sections were cut with an LKB Ultrotome III (LKB produkter AB Bromma Sweden) Areas containing terminal or respiratory bronchioles were first identified by light microscopic examination of 1 µ thick sections stained with toluidin blue Ultra thin sections from these areas were then stained with uranyl acetate and lead citrate and examined with a Jeol 100S electron microscope

Specimens for scanning electron microscopy (SEM) were also cut from the marginal portions of the lung Areas where the cut surface exposed the mucosa of longitudinally oriented terminal and preterminal con ducting airways were identified with a conventional dissection microscope The specimens were dehydrated with ethanol and Freon 113 followed by critical point drying They were coated with gold (100 Å) and examined with a Cambridge S4 scanning electron microscope with particular reference to the surface structure of the bronchiolar epithelium

As in the light microscopic study the evaluation of the specimens was made without knowledge of the experimental conditions

Statistical Evaluation

The Wilcoxon two sample test (two tailed) was used in our statistical calculations

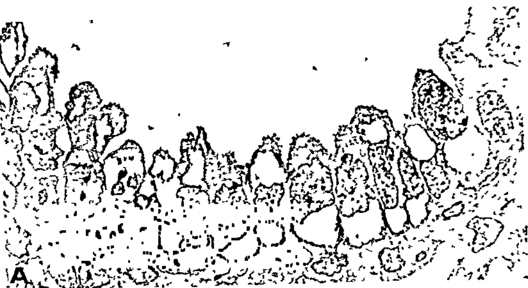


Fig 2 Ultrastructure of bronchiolar epithelium in non ventilated control fetuses showing immature glycogen rich Clara cells bulging into the lumen There is no evidence of epithelial necrosis
A TEM ×4 800
B SEM ×4 600

The exact pathogenesis of the bronchiolar epithelial lesions cannot be deduced from the present data but our morphological observations are concordant with the concept that these lesions reflect shear stress in the airway mucosa secondary to overdistension of preterminal conducting airways in the surfactant-deficient lung (*Hawker et al* 1967 *Robertson* 1976 *Nilsson et al* 1980 b) It is of particular interest that similar epithelial lesions develop in premature infants during the early course of IRDS *Gandy et al* (1970) noted such lesions in infants dying as early as 15 min after birth and



Fig 1 Light microscopic appearance of bronchiolar epithelial lesions induced by positive pressure ventilation Hematoxylin and eosin × 260

A Early lesions with pyknosis of individual epithelial cells This fetus was ventilated for 1 min

B More advanced lesions with pyknosis and desquamation of epithelial cells This fetus was ventilated for 5 min

C Prominent epithelial necrosis with hyaline membrane formation *(arrows)* in a small bronchiole after 30 min of artificial ventilation

Electron Microscopic Findings (TEM and SEM)

In fetuses killed *in utero* the bronchioles were lined by a regular epithelium, without evidence of necrosis or desquamation The epithelium dominated by non-ciliated cells containing amounts of cytoplasmic glycogen We interp these cells as immature Clara cells, as their su bulged into the bronchiolar lumen in a cobbles like pattern This pattern was a prominent fir in both SEM and TEM specimens Ciliated were comparatively scarce in the bronch mucosa (Fig 2)

Epithelial lesions were absent in all ventilated fetuses but present in all those subje to artificial ventilation

In general, the ultrastructural appearance of t lesions corresponded well with the light microsc findings described above As shown in Fig 3A 5A, the lesions were characterized by pyknosi individual cells, or small groups of cells with without desquamation Some of the desquam cells were flattened, elongated, and situated on surface of apparently intact cuboidal epithelial c (Fig 3B) These compressed necrotic cells w difficult to identify with certainty in SEM spe mens, as flattening of the epithelium is a norr feature in aerated bronchioles (Fig 4) Ot necrotic cells, scattered like grains over the muco surface, were easily recognized by SEM (Fig 5I

In addition to the bronchiolar lesions, fo necrosis and desquamation of the alveolar epit lium was occasionally observed in some fetus (Fig 6)

DISCUSSION

Our results show that bronchiolar epithelial lesior develop in premature newborn rabbits after only few minutes of positive pressure ventilation Thes findings are in agreement with earlier observation on premature newborn rhesus monkeys (*McAdam et al* 1973) or lambs (*Stahlman et al* 1964) I those series however, the methods for artificia ventilation were less standardized than in the present study

Judged by our morphometric findings the number of affected bronchioles does not increase after the first 10 minutes, although the character of the individual lesion does change Moreover, values for I_b representing the 30 min interval in the present study are in the same range as those obtained in previous experiments where the fetuses were ventilated under similar conditions for one hour (*Nilsson et al* 1978) A standardized ventilation period of 10 min has therefore been used in other experimental series in which we analyse the extent to which the development of epithelial lesions depends on variations in V_T and positive end expiratory pressure (*Nilsson et al* 1980 a, b)

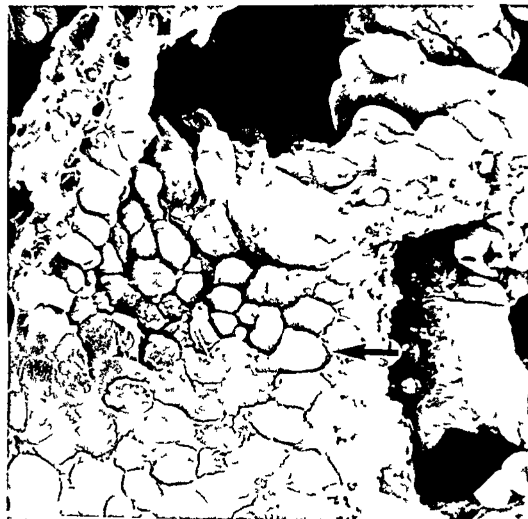


Fig 4 Fla ened ep hel al cells in the prox mal port on of a bronch ole aerated to the level nd cated by *arrow* The fetus was ventila ed for 5 m n but n th s f eld there is no conv nc ng ev dence of ep thel al necros s SEM × 2 400

morpholog cal equ valent of cl n cal IRDS) especi al y as this model allows recording of lung mechan cs during the course of the experiments

F nancial support was prov ded by the *Swed sh Med cal Resea h Counc l* project No 3351) the *Swed sh Na onal Assoc a on aga ns Hea and Ches D seases* and *K l s P edoc o al Research Fund* We thank *M Co er Fox* fo a s stance w h Engl sh

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˜ nun Two pyknotic epithelial cells are
type of lesion with multiple necrotic
9)

Finlay-Jones et al (1974) reported that necrosis of bronchiolar epithelium can occur »within a few minutes« after delivery even if the infant has only been gasping and no artificial ventilation has been applied

of
peripheral airways and *it* seems likely that bro
chiolar and alveolar hyaline membranes develop in areas where the epithelium has become at least partly desquamated and where the basement mem brane is denuded This hypothesis is supported by the observation that cell debris is a major compo nent of hyaline membranes (*Lauweryns* 1970) In view of this we share the opinion of *McAdams et al* (1973) that the artificially ventilated pre term fetus might serve as a useful experimental model of neonatal hyaline membrane disease (defined as the



Fig 6 Ultrastructure of alveolar epithelium in premature rabbit neonate ventilated for 5 min This field shows necrosis and desintegration of a membraneous pneumocyte TEM × 9 100

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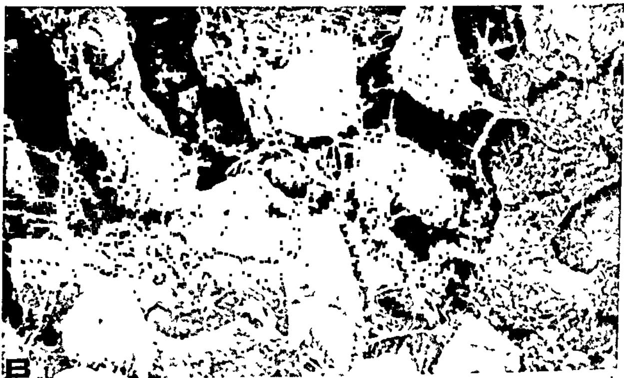


Fig 5 Desquamating bronchiolar epithelial cells in fetus ventilated for 5 min The necrotic cells have been › squeezed out of the epithelial lining and are scattered on the mucosal surface
A TEM ×6 000
B SEM ×5 600

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THE EFFECT OF FIXATION AND TRYPSINIZATION ON THE IMMUNOHISTOCHEMICAL DEMONSTRATION OF INTRACELLULAR IMMUNOGLOBULIN IN PARAFFIN EMBEDDED MATERIAL

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Jacobsen M Clausen P P & Smidth S The effect of fixation and trypsinization on the immunohistochemical demonstration of intracellular immunoglobulin in paraffin embedded material Acta path microbiol scand Sect A 88 369–376 1980

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was found for every 24 hours the fixation time was prolonged By comparing sections from tissue fixed in buffered formalin and selected fixatives (Lillie s AAF Bouin s fluid Clarke s fluid and 96% ethanol 1% acetic acid (E–A) processed at 4 °C and at 25 °C) an increased number of stained immunoglob [illegible]

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o [illegible] table immunoglobulin containing cells was investigated Trypsinization of sections from formalin fixed material increased the number of stainable cells substantially No essential effect was seen on tissue fixed in Lillie s AAF and Bouin s fluid In contrast trypsin treatment of sections from tissue fixed in Clarke s fluid and E–A completely destroyed the tissue No differences were observed between different immunoglobulin classes examined as regards the effect of fixation time fixatives and trypsinization

Key words Immunoglobulin immunoperoxidase fixation trypsinization lymphoid tissue

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Received 31 i 80 Accepted 4 vi 80

The demonstration of intracellular immunoglobulin on formalin fixed paraffin embedded tissue by the immunoperoxidase staining method was first described by *Taylor & Burns* in 1974 (22) The method has later on be[illegible] from [illegible] reactiv[illegible] [illegible] lymph[illegible] tissue with neoplastic or reactive conditions (1 8 9 10 21)

The results described in these investigations have however not always been concordant and reproducible (10 24) It is only possible to compare results based on material which has been prepared in different ways if information is available regarding the influence of the tissue preparation on the ant[illegible] [illegible]

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Using an indirect labelled immunoperoxidase technique the influence of fixation time on the antigenicity of intracellular immunoglobulin in lymphoid tissue fixed in buffered formalin has been investigated Within a fixation period of 96 hours a decrease of 15% of stainable immunoglobulin containing cells was found for every 24 hours the fixation time was prolonged By comparing sections from tissue fixed in buffered formalin and selected fixatives (Lillie s AAF Bouin s fluid Clarke s fluid and 96% ethanol 1% acetic acid (E–A) processed at 4 °C and at 25 °C) an increased number of stained immunoglobulin containing cells was found in tissue fixed in Lillie s AAF Bouin s fluid Clarke s fluid and E A processed at 4 °C No difference was found between tissue fixed in buffered formalin and E–A processed at 25 °C In addition the effect of pretreatment of the sections with trypsin on the number of stainable immunoglobulin containing cells was investigated Trypsinization of sections from formalin fixed material increased the number of stainable cells substantially No essential effect was seen on tissue fixed in Lillie s AAF and Bouin s fluid In contrast trypsin treatment of sections from tissue fixed in Clarke s fluid and E–A completely destroyed the tissue No differences were observed between different immunoglobulin classes examined as regards the effect of fixation time fixatives and trypsinization

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The demonstration of intracellular immunoglobulin on formalin fixed paraffin embedded tissue by the immunoperoxidase staining method was first described by *Taylor & Burns* in 1974 (22) The method has later on been employed on materials from multiple myeloma macroglobulinaemia and reactive plasmocytosis (5 17 23 24) as well as on lymphatic tissue with neoplastic or reactive conditions (1 8 9 10 21)

The results described in these investigations have however not always been concordant and reproducable (10 24) It is only possible to compare results based on material which has been prepared in different ways if information is available regarding the influence of the tissue preparation on the antigens to be demonstrated

The aim of the present investigation is to examine the influence of fixation time and selected fixatives on the demon[illegible]

[illegible] with trypsin has been evaluated

MATERIAL AND METHODS

Tissues

Lymph nodes removed during cholecystectomia colectomia and hysterectomia were used

Immediately following the removal the nodes were divided in two parts One part (*the reference specimen*) was always fixed for 4 hours in 10% phosphate buffered *formalin at room* temperature The other part (the test specimen) was treated according to one of the following procedures

A Fixation in 10% buffered formalin at room *temperature for 4 8 12 18 24 48 72* and 96 hours

B Fixation for 1 4 8 24 *hours at room temperature* in the following fixatives Lillie s AAF (10 ml 30% *neutral* formaldehyde solution 5 ml glacial acetic acid 85 ml 99% ethanol) Bouin s fluid (75 ml 1 2% picric acid aqueous solution 25 ml neutral 30% formaldehyde solution 5 ml glacial acetic acid) and Clarke s fluid (80 ml 99% ethanol 20 ml glacial acetic acid)

C Fixation for 24 hours at 4 °C *in 96% ethanol 1%* glacial acetic acid (E–A)

Following the fixation tissues treated according to A and B were dehydrated in ethanol and xylene at room temperature and embedded in Paraplast® at 58–60 °C Tissues fixed according to C were either processed as A and B or dehydrated at 4 °C until the last change of xylene after which the temperature was increased to 25 °C and the material was then embedded as described *above (19) In cases where the tissue could not* be embedded following the dehydration tissue was preserved in *70%* ethanol for a maximum of *18* hours until further dehydration

5 µm serial sections were cut

Trypsinization

In order to examine the effect of pretreatment of the sections with trypsin on the number of stained *immunoglobulin containing cells dewaxed sections from lymph nodes fixed for 24 hours in the above mentioned* fixatives were incubated for 5 20 min with 0 1% trypsin (Sigma type III) 0 1% calcium choride in distilled water adjusted to pH 7 8 with 0 1 M NaOH (14)

Antisera

Following antisera and lots were used anti lambda (*18B*) *anti kappa* (*18 C*) *anti α* (*18 B*) *anti γ* (*28 C*) and anti µ (88 C 18 A) (DAKO Denmark) and anti δ (1102 C 1102 L 1104 B) *and anti ε* (*1203 E 1203 D*) (Behringewerke Hoechst Denmark)

The specificity of the antisera was verified by performance testing on formalin fixed paraffin embedded material from multiple myeloma of known monoclonal *protein type*

Working dilutions were found by titration Anti lambda *and anti kappa* were used in dilutions 1/100–1/102400 anti α γ and µ in dilutions 1/40–1/20480 anti δ and ε in dilutions *1/5–1/2560*

Peroxidase
lin (lot 28 A 3
dilution 1/20

For control *stainings the immunoglobulin* fraction of serum from unimmunized rabbits (lot 38 B) (DAKO) was used in *dilution 1/80* (corresponding to the maximum protein concentration of the used antisera)

All antisera were diluted in phosphate buffered saline (PBS) pH 7 2

Staining Procedure

Each set of lymph nodes was stained for lambda kappa α γ µ δ and ε chains

Indirect peroxidase labelled antibody technique (6) *was used*

As control stainings the specific antisera were replaced by 1) immunoglobulin fraction of serum of *unimmunized rabbits 2) PBS In addition the staining of* different cell populations by using different antisera served as an inherent control

In each staining series serial sections from both parts of each lymph node were stained for all the immunoglobulins mentioned

Counting Method

The number of stained cells was determined by counting all cells with positive intracytoplasmic staining reaction in every second counting unit defined by the
[illegible] 10 × 10 mm 100
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per unit was calculated *In* order to evaluate the reproducibility of the counting method used the same *section was counted five times and the variation* coefficient was calculated to 2% the variation from one serial section to another was less than 4% and the variation from one part of the lymph node to the other part identically processed was less than 4%

Evaluation

Evaluation of the influence of the fixation time on the *number of stained cells was made by expressing* the results as a percentage between the number of cells *in the* test section and in the reference section

The statistical evaluation of corresponding values of fixation time and number of stained cells was made by means of correlation and regression tests When comparing regression equations the F test of variance was used The limit for type 1 error was set at 0 05

Evaluation of the effect of the different fixatives on the number of stained cells compared to the effect of *buffered formalin was performed* by counting the number of cells in the sections related to increasing dilution of the primary antisera *The number of cells* was semiquantitatively evaluated according to the following scoring system *0* + ++ +++ and ++++ (+ indicating up to 20 cells per unit ++ up to 40 cells per unit +++ up to 60 cells per unit and ++++ more than 60 cells per unit)

A comparative evaluation of the different fixatives at constant fixation time and the effect of pretreatment with trypsin was made by comparing the dilutions which gave the same number of stained cells in the reference section and test section

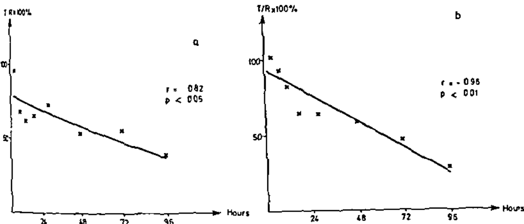


Fig 1 a–b The effect of fixation time on the number of stained immunoglobulin containing cells in formalin fixed paraffin embedded tissue (T = number of cells in test section R = number of cells in reference section) a) light chains b) heavy chains

Fig 2 a–d The effect of selected fixatives on the number of stained immunoglobulin (kappa) containing cells in relation to antiserum dilution Comparison between four hours formalin fixation and four hours fixation in a) Lillie's AAF b) Bouin's fluid c) Clarke's fluid and d) 24 hours *ethanol acetic* acid processed either at 4 °C or 25 °C

MATERIAL AND METHODS

Tissues

Lymph nodes removed during cholecystectomia, colectomia and hysterectomia were used

Immediately following the removal, the nodes were divided in two parts One part (the reference specimen) was always fixed for 4 hours in 10% phosphate buffered formalin at room temperature The other part (the test specimen) was treated according to one of the following procedures

A Fixation in 10% buffered formalin at room temperature for 4, 8, 12, 18, 24, 48 72 and 96 hours

B Fixation for 1, 4, 8, 24 hours at room temperature in the following fixatives Lillie's AAF (10 ml 30% neutral formaldehyde solution, 5 ml glacial acetic acid 85 ml 99% ethanol), Bouin s fluid (75 ml 1 2% picric acid aqueous solution, 25 ml neutral 30% formaldehyde solution, 5 ml glacial acetic acid) and Clarke's fluid (80 ml 99% ethanol, 20 ml glacial acetic acid)

C Fixation for 24 hours at 4 °C in 96% ethanol, 1% glacial acetic acid (E–A)

Following the fixation tissues treated according to A and B were dehydrated in ethanol and xylene at room temperature and embedded in Paraplast® at 58–60 °C Tissues fixed according to C, were either processed as A and B or dehydrated at 4 °C until the last change of xylene, after which the temperature was increased to 25 °C and the material was then embedded as described above (19) In cases where the tissue could not be embedded following the dehydration tissue was preserved in 70% ethanol for a maximum of 18 hours until further dehydration

5 µm serial sections were cut

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In order to examine the effect of pretreatment of the sections with trypsin on the number of stained immunoglobulin containing cells dewaxed sections from lymph nodes fixed for 24 hours in the above mentioned fixatives were incubated for 5–20 min with 0 1% trypsin (Sigma, type III) 0 1% calcium choride in distilled water, adjusted to pH 7 8 with 0 1 M NaOH (14)

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The number of stained cells was determined counting all cells with positive intracytoplasmic staini reaction in every second counting unit, defined by th outer frame of an ocular grid (Leitz 10 × 10 mm 10 squares) with 25 × objective and 10 × ocular Not les then 25 units were counted (3) and the number of cel per unit was calculated In order to evaluate th reproducibility of the counting method used the sam section was counted five times and the variatio coefficient was calculated to 2%, the variation from on serial section to another was less than 4% and th variation from one part of the lymph node to the othe part identically processed was less than 4%

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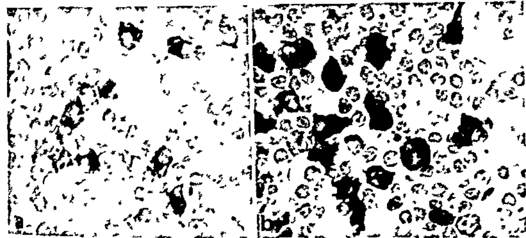


Fig 4 a–b a) Immunoperoxidase staining for IgG of a lymph node fixed in buffered formalin for 24 hours (Counterstained with hematoxylin × 800) b) Adjoining section stained as a with trypsinization of the section before staining (Counterstained with hematoxylin × 800)

The Effect of Trypsinization

Trypsin treatment of sections from tissue fixed in Clarke's fluid and E–A completely destroyed tissue morphology Trypsinization of sections fixed in Bouin's fluid and Lillie's AAF had no essential influence on tissue morphology or immunoglobulin staining In contrast trypsinization of sections from formalin fixed material showed a pronounced effect on the immunoglobulin staining Tissue morphology did not change but the number of stained cells was considerably increased and the staining reaction in trypsin treated sections was more uniform and finely granular (Fig 4 b) Different immunoglobulin classes showed the same reaction pattern

Following trypsin treatment of formalin fixed tissue immunoglobulin on the surface of the small lymphocytes was to some extent demonstrable and the staining of the connective tissue fibers was slightly more pronounced

DISCUSSION

Until now only few papers have systematically dealt with the influence of fixation time on the preservation of antigenicity *Kraehenbuhl* (15) using glutaraldehyde and formaldehyde described a loss of antigenicity of various antigens including IgA of up to 40% during the first 24 hours of the fixation period Using formalin as fixative we have likewise for all classes of immunoglobulins demonstrated a high degree of correlation between decrease of stained cells and fixation time

Thus comparing results obtained by immunohistochemical staining for immunoglobulin in formalin fixed material knowledge of the fixation time used is essential

The effect of different fixatives on the antigenicity of intracellular immunoglobulin has until recently only been investigated to a limited extent Table 1 presents a survey of results published within the latest five years including our own results

Our results are in general in accordance with the previously published results In addition to Bouin's fluid we found that Lillie's AAF gave good preservation of antigenicity of immunoglobulins sharp demarcation of the staining reaction as well as satisfactory preservation of tissue morphology Clarke's fluid likewise gave good antigen preservation but tissue morphology was less satisfactory Using fixation in E–A at 4 °C and cold processing of the tissue in accordance with *Sainte Marie* (19) resulted in moderate to good antigen preservation while routine processing gave a less satisfactory result In both cases however tissue morphology was bad and background staining intense

The employment of digestion with proteolytic enzymes on sections from formalin fixed and paraffin embedded material was introduced by *Huang* (13) [illegible]

[illegible] unofluorescence as by immunoperoxidase technique

The treatment has been described to result in unmasking of immunoreactive sites with increased number of stained cells demonstration of surface

RESULTS

The influence of the fixation time on the number of stained cells in formalin fixed material is shown in Fig 1 a and b The number of stained cells in tissue fixed in formalin for varying time period (T) is related to the number of stained cells in tissue fixed in formalin for 4 hours (R) The number of cells stained for δ and ε chains was to small to be taken into account As the regression equations for the two light chains (kappa lambda) and the three heavy chains (α γ μ) respectively showed no statistical differences the results for the light chains and for the heavy chains were pooled

It is seen that within the fixation time examined there was a decrease of stained cells of 10–20% for every 24 hours the fixation time was prolonged

The influence of selected fixatives compared to the influence of formalin on the number of stained cells is shown in Fig 2 a–d All the lymph nodes have been fixed for 4 hours (E–A for 24 hours at 4 °C) and the number of stained cells is correlated to increasing dilution of the primary antisera

It is seen that tissue fixed in Lillie s AAF Bouin s *fluid Clarke s fluid and E–A followed by cold* processing at all dilutions contained more stained cells than tissue fixed in buffered formalin In contrast no substantial difference in the number of *stained cells was found in tissue fixed in E–A* followed by routine processing compared to tissue fixed in buffered formalin The different immuno globulin classes showed the same pattern of reaction

In addition to quantitative differences differences in regard to tissue morphology and quality of the immunoperoxidase staining were found (summarized in Table 1) The morphology of the tissue was good when using Lillie s AAF Bouin fluid and buffered formalin while fixation in E–A and Clarke s fluid lead to a rather pronounced shrinkage of the cells increasing with prolonged fixation time

The intracytoplasmic staining was homogeneous granular with well defined cell borders when using Lillie s AAF Bouin s fluid Clarke s fluid and E–A (Fig 3) In buffered formalin a rough granular staining with heterogeneous distribution and ill defined cell borders was found and this was more pronounced when longer fixation time was used (Fig 4 a)

Staining of the extracellular immunoglobulin and surface immunoglobulin on small lymphocytes was to some extent demonstrable when the tissue was fixed in Lillie s AAF Clarke s fluid or E–A but not in tissue fixed in Bouin s fluid and buffered *formalin*

Staining of the connective tissue fibers and background for especially IgG was most pronounced in formalin Clarke s fluid and E–A fixed tissue *whereas Bouin s fluid and Lillie s AAF fixed tissue* showed a weaker connective tissue and background staining

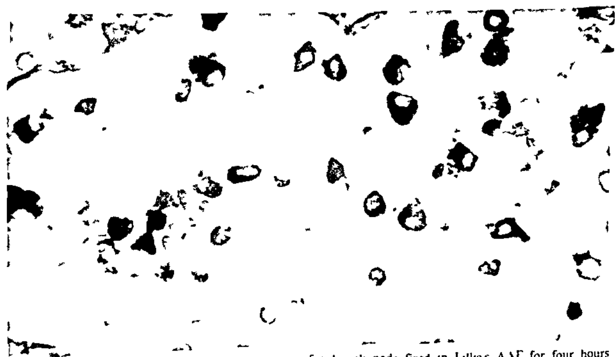


Fig 3 Immunoperoxidase staining for kappa chains of a lymph node fixed in Lillie s AAF for four hours (Counterstained with hematoxylin × 800)

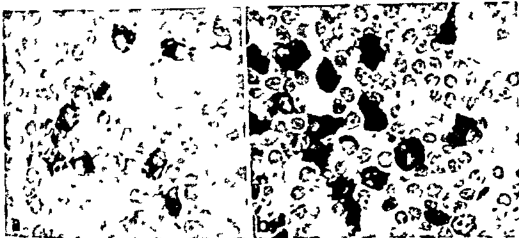


Fig 4 a–b a) Immunoperoxidase staining for IgG of a lymph node fixed in buffered formalin for 24 hours (Countersta ned w th hematoxylin × 800) b) Adjoining section sta ned as a, with trypsinization of the section before stain ng (Countersta ned with hematoxylin × 800)

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The employment of digestion with proteolytic enzymes on sections from formalin fixed and paraffin embedded material was introduced by *Huang* (13) later on trypsin (7 8 14) pronase (11) and pepsin (18) have been used in the demonstration of immunoglobulin in paraffin sections as well by immunofluorescence as by immunoperoxidase tech nique

The treatment has been described to result in unmasking of immunoreactive sites with increased number of stained cells demonstration of surface

TABLE 1 *Survey of the Results Obtained by Immunohistochemical Staining for Immunoglobulin in Tissue Fixed in Various Fixatives*

Reference	Fixative	Fixation temp (C)	Hours	Preservation of antigenicity	Definition of staining reaction	Background staining	Tissue morpholo
Burns et al (1974)	Abs ethanol	4°	24	++	++	strong	++
	Formol saline 10%			+++	+++	moderate	+++
Kuhlmann (1976)	Buffered formaldehyde 4%	4°	24	+			
	Buffered formaldehyde 1% + glutaraldehyde 1%	4°	1–1 5	+			
	Glutaraldehyde 1–1 5%	4°	1–1 5	+			
	Ethanol 96% + acetic acid 1%		10–15	++			
Bosman et al (1977)	Buffered formalin 10%	25°	3	+			+++
	Bouin s fluid	25°	3	++	+++	weak	+++
	Sublimate formaldehyde	25°	3	+++	+++	moderate	++(<20 ho
	Formalin 10% + glutaraldehyde 6%	25°	3	0			+
	Zenker s fluid	25°	3	+	+	strong	+++
	Buffered methanol formalin	25°	3	+			+++
	Periodic acid lysine paraformaldehyde	25°	3	++	+	moderate	+++
Dorsett et al * (1977)	Bouin s fluid	25°	6–8	+++	+++	weak	+++
Curran et al (1977)	Buffered paraformaldehyde 4%	4–20°	24	+			
	Buffered formaldehyde 4%	4–20°	24	+			
	Formol saline 10%	4–20°	24	++			
	Aqueous formaldehyde 4%	4–20°	24	+++			
	Formalin 10% + glutaraldehyde 0 5% + calcium acetate 2%	4–20°	2–24	+(<2 hours)	+++	strong	+++
	Ethanol 95%	4°	24	+	+	strong	+
	Diethylpyrocarbonate	55°	3	+++	+++		+++
Jacobsen et al present results	Buffered formalin 10%	25°	1–96	+	+	moderate	+++
	Lillie s AAF	25°	1–96	+++	+++	weak	+++
	Bouin s fluid	25°	1–96	+++	+++	weak	+++
	Clarke s fluid	25°	1–96	+++	+++	strong	+
	Ethanol 96% acetic acid 1% (routinely processed)	4°	24	+	+	strong	+
	Ethanol 96% acetic acid 1% (processed at 4°)	4°	24	+++	+	strong	+

+++ good ++ moderate + poor 0 extremely bad * Immunofluorescence

immunoglobulin on small lymphocytes and suppression of background staining to some degree

In accordance with these observations we have found that trypsin digestion of formalin fixed material caused increased number of demonstrable immunoglobulin containing cells just as surface staining on small lymphocytes to some extent could be revealed No reduction of background staining was observed

The mechanism of the demasking effect of trypsinization has not yet been completely clarified As explanations increased permeability of the cells (7) or cleavage of cross links between fixative and antigenic determinants (8 14) have been suggested The latter hypothesis is supported by investigations performed by *Takamiya et al* (20) who showed that antigenic determinants could be masked by modification of amino groups with

benzyl chloroformate and demasked by treatment with protease

Our results lend support to the latter hypothesis ... had only

only when trypsin digestion was used for a limited period Trypsinization of material fixed in fixatives without formalin caused digestion of the tissue just as trypsinization of formalin fixed material for prolonged periods

In conclusion we want to stress the importance of specifying the fixation time when comparing different results obtained by staining for immunoglobulin on formalin fixed material

In order to obtain a result combining high amount of stained immunoglobulin containing cells with acceptable tissue morphology on paraffin embedded material we suggest fixation in Bouin s fluid or Lillie s AAF It should however be mentioned that Bouin s fixation is not compatible with the use of Methyl Green Pyronin staining for nucleic acids which sometimes is used in characterization of lymphoid cells in tissue sections Formalin fixation within a limited period is usable and the masking of stainable immunoglobulin containing cells when prolonged fixation has been used can to a great extent be broken by controlled trypsinization of the section

We wish to thank mrs *Britta Arbjørn* for carefully typing the manuscript and mr *Aksel Ankerstjerne* for his technical assistance

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benzyl chloroformate and demasked by treatment with protease

Our results lend support to the latter hypothesis as we have found that trypsinization had only positive or at least no negative effect on the staining result, when using formalin containing fixatives and only when trypsin digestion was used for a limited period Trypsinization of material fixed in fixatives without formalin caused digestion of the tissue just as trypsinization of formalin fixed material for prolonged periods

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ENZYMECYTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY IN MONOCLONAL GAMMOPATHY AND REACTIVE PLASMACYTOSIS

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Johansen P & Krogh Jensen M Enzymecytochemistry and immunohistochemistry in monoclonal gammopathy and reactive plasmacytosis Acta path microbiol scand Sect A 88 377–382 1980

Peripheral blood smears and bone marrow smears from 29 patients with malignant M components (25 with multiple myeloma and 4 with malignant lymphoma) 13 patients with benign monoclonal gammopathy (BMG) and 20 patients with polyclonal reactive plasmacytosis were examined by leucocyte alkaline phosphatase score (LAP-score) and by acid phosphatase score in plasma cells from bone marrow smears Furthermore tissue sections from marrow biopsies from all patients were examined by the three layer unlabelled immunoperoxidase technique to detect cytoplasmic immunoglobulin The LAP score was significantly higher in patients with malignant M-components than in patients with BMG and also higher in IgA and IgG myeloma than in IgA and IgG BMG but the latter difference was not significant Furthermore a significant positive correlation between paraprotein concentration and LAP score was found in multiple myeloma Acid phosphatase score in plasma cells showed no clear distinction between multiple myeloma and BMG Immunohistochemical examination showed a distinct monoclonal pattern in both multiple myeloma and BMG allowing identification of the M-component which in all cases corresponded to the M-component detected by serum examination Cells producing immunoglobulin classes not matching the M-component were more rare in multiple myeloma than in BMG but the difference between the two conditions was quantitative and allowed no clear distinction

Key words Monoclonal gammopathy enzymecytochemistry immunohistochemistry

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Prevalence studies have shown that the frequency of serum M-components in the population increases with age In individuals over the age of 80 approximately 5% have M-components (1) The discovery of an M-component always brings about examinations to exclude or confirm a diagnosis of multiple myeloma or malignant lymphoma In the absence of heavy bone marrow infiltration osteolytic skeletal lesions or enlarged lymphoid organs differential diagnosis is difficult between a malignant disease of plasmacytic or lymphocytic origin and the clinical syndrome benign monoclonal gammopathy [illegible] t criteria such as the concentration of the monoclonal immunoglobulin fraction presence of Bence Jones protein and depression of other immunoglobulin classes are poor discriminating parameters (9)

Enzymecytochemical studies of plasma cells have demonstrated an increased activity of acid phosphatase in multiple myeloma compared to normal and reactive plasma cells (10 11) Furthermore semi quantitation of cytoplasmic acid phosphatase activity in plasma cells from cases of multiple myeloma and BMG has demonstrated a significantly higher activity of this enzyme in multiple myeloma facilitating a clear distinction between the two conditions (4) Leucocyte alkaline phosphatase

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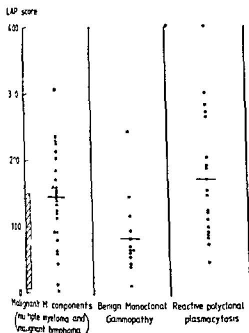


Fig 1 LAP score levels in malignant and benign monoclonal gammopathy and reactive plasmacytosis The following signatures are used to identify individual immunoglobulin classes △IgA □IgG ○IgM × light chain disease The hatched area on the interval scale represents the range of LAP scores in the healthy controls

RESULTS

Fig 1 illustrates the LAP-score levels The range of LAP-scores from the healthy controls is shown by the hatched area on the interval scale Patients with reactive polyclonal plasmacytosis show a high mean LAP score with a considerable variation in individual scores The mean LAP score in patients with malignant M-components is 146 with a range from 7 to 308 while the mean score in patients with BMG is 87 with a range from 15 to 244 this difference being significant ($P<0.01$) The LAP score in patients with multiple myeloma of IgA and IgG type is higher than the LAP-score in patients with BMG of the same types but the difference is not significant In the BMG group patients with serum M-components of IgM type tended to have lower LAP scores than patients with M-components of IgA and IgG type Of the four patients with a malignant M-component of IgM type all had malignant lymphomas the score was elevated in two and in two within the range of the healthy controls Fig 2 illustrates the correlation between serum paraprotein concentration and LAP-score in patients with multiple myeloma and shows a positive correlation ($r=0.58$) between the two parameters this correlation being significant ($P<0.01$) In patients with BMG of IgA and IgG type LAP-scores also increased with increasing paraprotein concentration while patients with BMG of IgM type showed no correlation between LAP score and paraprotein concentration All patients with monoclonal gammopathy had normal leucocyte counts at the time of the LAP-score determination Three patients with multiple myeloma received therapy at the time of blood and bone marrow examination the LAP-score levels in these cases were all within the range of LAP scores from the controls

The acid phosphatase scores are illustrated in

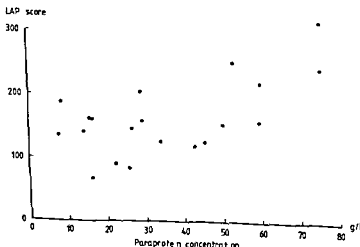


Fig 2 Correlation between LAP-score and paraprotein concentration in patients with multiple myeloma ($r=0.58$ $p<0.01$)

activity in neutrophils is also reported to be elevated in patients with multiple myeloma (3 12) but information is lacking concerning the level of this enzyme in patients with BMG

Immunocytochemical investigations with special reference to the *percentage of cells* producing the immunoglobulin matching the serum M component have not revealed a clear distinction between patients with a malignant disease of plasmacytic or lymphocytic origin and BMG (6 15)

The purpose of this study was to test the difference in cytoplasmic acid phosphatase activity between *plasma cells of multiple* myeloma and BMG and to see if the level of leucocyte alkaline phosphatase activity (LAP score) allowed a distinction between multiple myeloma and BMG Furthermore bone marrow biopsies were examined by immunoperoxidase technique to evaluate the distribution of immunoglobulin producing cells by an *immunohistochemical method in these conditions*

MATERIAL AND METHODS

The material consists of 62 consecutive patients from whom peripheral blood smears bone marrow smears and bone marrow biopsies were examined during a 12 months period (February 1 1978 to January 31 1979) The criteria for entering the study were 1) presence of an M *component in serum and/or urine* and 2) bone marrow smears with an increased number of plasma cells *(more than 3% of nucleated marrow cells)* The [illegible] three gro ps 1) mal gnant M

sis (20) *Multiple myeloma* was *diagnosed when at least* two of the following three criteria were present 1) M *component in serum and/or urine 2) osteolytic* skeletal lesions and 3) bone marrow smears with more than *15% plasma cells and/or tissue sections from marrow* biopsies showing large solid plasma cell infiltrates The diagnosis *of malignant lymphoma* was *based on* histologic examination of lymphatic tissue (lymph nodes and spleen) *Cases with serum M components of IgA and* IgG type not fulfilling the criteria for multiple myeloma and cases with *IgM serum M components without* enlarged lymphoid organs were classified as BMG patients in this group *have been followed for at least one* year without significant increase in the serum M component *Reactive plasmacytosis* was *diagnosed in* patients without a serum M component but with more tha [illegible] marrow smears

in [illegible]
for alkaline phosphatase according to [illegible]
(7) *One hundred consecutive neutrophils* were counted and the intensity of enzyme activity was graded from 0 to ++++ Simultaneously *with each* blood smear from a patient a smear from a healthy control was stained and scored Bone marrow *smears were air* dried and stained for acid phosphatase (8) In patients with multi myeloma BMG with IgA and IgG M *components* a reactive plasmacytosis the intensity of acid phosphat activity in individual plasma cells *was graded by* semiquantitative scoring system from 0 to ++++ T weakest reaction consisted *of a diffuse rose cytoplasm* tint with increasing activity a granular reaction occu red the *strongest reaction consisted of* confluе granular positivity In each smear the cytoplasm *activity of acid phosphatase* was registered in 1(consecutive plasma cells and the result is referred to *the acid phosphatase score* Scoring was not performed cases with less than 100 plasma cells in the bone marro smear

Tissue sections from formalin fixed paraffin embec ded bone marrow biopsies (marrow clots or iliac cre biopsies) were examined by the three layer unlabelle immunoperoxidase (PAP) method described in deta elsewhere (5) The method consists of the followin steps 1) sections are deparaffinized and brought t absolute alcohol 2) endogenous peroxidase activity i blocked with H_2O_2 in methanol solution 3) sections ar incubated for 30 min with rabbit antihuman immuno globulin antisera Working dilutions of antisera in this step were found by titration In the demonstration of heavy chains concentrations from 1/81 to 1/1001 were used for the demonstration of light chains dilutions from 1/81 to 1/2001 were used 4) incubation with swine antirabbit immunoglobulin in dilution 1/41 5) incubation with rabbit peroxidase antiperoxidase (PAP) complex in dilution 1/101 6) peroxidase activity is developed by incubation with 0 05% 3 3 diaminobenzidintetrahydrochloride (Sigma) and 0 01% H_2O_2 in 0 1 M phosphat buffered saline pH 7 6 In steps 3 4 and 5 antisera contained 20% normal swine serum Antisera were diluted in phosphate buffered saline pH 7 2 All sections were examined with antisera against human alpha gamma my heavy chains lambda and kappa light chains Cases of multiple myeloma without alpha or gamma heavy chains were also examined for the presence of delta and epsilon heavy chains Antisera against human delta and epsilon heavy chains were purchased from Behringwerke Hoechst Denmark all other antisera were purchased from DAKO Denmark In all cases control sections were made by 1) incubation with the immunoglobulin fraction of serum from unimmunized rabbits instead of specific rabbit antiserum 2) incubation with PBS instead of specific rabbit antiserum Furthermore the varying number of cells stained with each antibody acted as inherent controls All sections were counterstained with haematoxylin and mounted in Pertex® (Histolab Bethlehem Trading Ltd)

In each patient the leucocyte count at the time of LAP score determination was recorded

Qualitative identification of the M component in serum was based on immunoelectrophoresis and the serum concentration of all M components was measured (2)

Statistic analysis of differences in score levels was made by the Mann Whitney test



Fig 4 Immunoperoxidase staining of marrow biopsy from a case of BMG with a serum IgA kappa M component showing scattered positive cells (arrows) and a focal infiltrate (upper right) 3 layer unlabelled immunoperoxidase technique for IgA haematoxylin counterstaining × 512

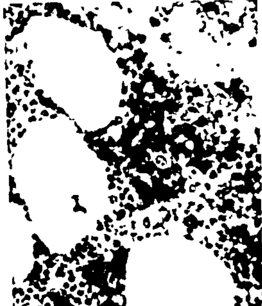


Fig 6 Immunoperoxidase staining of marrow biopsy for IgG from a case of IgG kappa myeloma showing heavy infiltration with only small uninvolved areas 3 layer unlabelled immunoperoxidase technique for IgG haematoxylin counterstaining × 512



Fig 5 Same case as Fig 4 stained for IgG showing scattered positive cells (arrows) 3 layer unlabelled immunoperoxidase technique for IgG haematoxylin counterstaining × 512

for this elevated LAP-score is unknown Intercurrent infectious diseases known to cause an elevated LAP score seem unlikely as we find normal leucocyte counts in all patients with M-components corresponding to the findings of *Brook and Dreisbach* (3) In this study we find a significant positive correlation between serum paraprotein concentration and LAP score in patients with multiple myeloma supporting the observation by *Überla et al* (16) who found a positive correlation between LAP score and serum gamma globulin concentration The reason for this correlation is not known but the finding is of practical importance as the determination of LAP scores in patients with M components seems to give no further information than can be achieved by determination of serum paraprotein concentration However it still needs to be known whether an elevated LAP-score precedes a rise in paraprotein concentration Therefore a patient with an M-component and a high LAP score but without sufficient evidence of multiple myeloma should be followed closely

Immunocytochemical examination of bone marrow smears in monoclonal gammopathy using immunofluorescence technique has demonstrated a

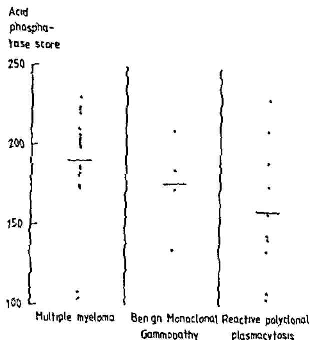


Fig 3 Acid phosphatase score in multiple myeloma benign monoclonal gammopathy with IgA and IgG M-components, and reactive plasmacytosis

Fig 3 The highest mean score is found in patients with multiple myeloma but a considerable overlapping between the three groups exists and the differences in acid phosphatase score are insignificant The acid phosphatase score did not correlate to serum paraprotein concentration

Immunohistochemistry

In reactive plasmacytosis the immunoperoxidase-stained bone-marrow sections invariably showed a polyclonal pattern with the presence of an equal number of IgA and IgG producing cells and rare IgM producing cells In the presence of a serum M-component, whether multiple myeloma or BMG, the immunoperoxidase-stained marrow sections disclosed a plasma cell population, where approximately 80 % or more of the cells produced the same immunoglobulin class or the same light chain which always corresponded to the serum and/or urine M-component In cases of BMG with an IgM serum M component immunoperoxidase staining showed a population of lymphoplasmacytoid cells positive for IgM These cells occurred singly and in small groups In multiple myeloma, plasma cells producing immunoglobulin not matching the serum M component were rare while in BMG such cells were more numerous up to approximately 20 % (Fig 4 and 5) However, marrow sections from cases of BMG sometimes contained very few cells not matching the serum M-component In multiple myeloma plasma cell infiltrates tended to be more extensive (Fig 6) but owing to the relative small samples of marrow-tissue plasma cell infiltrat[illegible] cases of multiple myeloma could be discrete [illegible] these reasons the differences between mul[illegible] myeloma and BMG were only quantitative [illegible] allowed no clear distinction In three of the [illegible] cases of malignant lymphoma the bone-mar[illegible] was infiltrated, and the cytoplasm of the lymp[illegible] plasmacytoid cells contained monoclonal immu[illegible] globulin matching the serum M-component In [illegible] of the three cases, marrow involvement [illegible] minimal but the monoclonal cytoplasmic immu[illegible] globulin facilitated the recognition of marrow in[illegible] tration from malignant lymphoma

DISCUSSION

Enzymecytochemical studies of normal and reacti[illegible] plasma cells and plasma cells in multiple myelon[illegible] have revealed an increased activity of acid phosph[illegible] tase in the latter condition (10, 11) This differen[illegible] is of little value in clinical practise, as apart fro[illegible] rare cases of nonsecretory myeloma, the differenti[illegible] diagnosis between multiple myeloma and reacti[illegible] plasma cell hyperplasia is easily made by serolog[illegible] examination Of considerable interest is, however, [illegible] study by *Cassuto et al* (4) who found a significar[illegible] difference between multiple myeloma and BMG a[illegible] regards cytoplasmic activity of acid phosphatase [illegible] plasma cells We, however, cannot confirm thi[illegible] observation Though we have been able to quanti[illegible] tate only a few cases of BMG, our results show [illegible] considerable overlapping between the values in th[illegible] two groups The mean score is higher in multipl[illegible] myeloma but the difference is not significant N[illegible] major differences seem to exist between the enzymecytochemical technique in our study and th[illegible] one by *Cassuto et al* (4), but minor differences ma[illegible] be found in the criteria used to separate multipl[illegible] myeloma and BMG, as Cassuto et al (4) do not specify the criteria in their paper The cause of the elevated, but in our study not significantly higher, acid phosphatase activity in plasma cells from multiple myeloma is unknown We find as *Löffler* (11) no correlation between serum paraprotein concentration and acid phosphatase activity

We find a significantly higher LAP-score level in patients with malignant M-components than in cases of BMG In our study this difference is due to the facts that patients with BMG of IgM type all have a LAP-score within the range of the healthy controls and that the LAP score in patients with multiple myeloma of IgG and IgA type is higher than in patients with IgG and IgA BMG The LAP-score has previously been reported to be elevated in patients with multiple [illegible] (3, 12) The reason

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THE ANTRAL GASTRIN-PRODUCING CELLS IN DUODENAL ULCER PATIENTS

A Density Study before and during Treatment with Cimetidine

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Nielsen H O Jensen K B & Christiansen L A The antral gastrin producing cells in duodenal ulcer patients A density study before and during treatment with cimetidine Acta path microbiol scand Sect A 88 383–386 1980

A density study of the antral gastrin producing cells has been performed before and after eight weeks of treatment with a histamine H_2 receptor antagonist (cimetidine 1 g per day) in a series of 38 patients suffering from chronic duodenal ulcer The treatment produced a significant increase in numerical density and a decrease in mean cell volume The volume density of the cells was unchanged suggesting

[illegible]

[illegible] discontinuation of the treatment

Key words Duodenal ulcer cimetidine gastric acid secretion serum gastrin gastrin-cell density

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The treatment of chronic duodenal ulcer by selective vagotomy or histamine H_2 receptor antagonists is followed by inhibited basal pentagastrin and food stimulated gastric acid secretion (2 9 21) and by elevated levels of basal and food stimulated serum gastrin (6 10 22) As hypo and achlorhydria may cause elevated serum gastrin levels and gastrin-cell (G-cell) hyperplasia (12 17) it is logical to suggest that the treatment of duodenal ulcer as used today can increase the G-cell population

Becker et al (5) and *Delince et al* (8) in contrast to *Creaghe et al* (7) found that vagotomy performed on rats resulted in a hyperplasia of the antral G cells *Witt et al* (27) found no change in volume density of the G-cel [illegible]

[illegible]

during treatment with a histamine H_2-receptor antagonist cimetidine

MATERIAL AND METHODS

The [illegible]

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(range 31 to 76 years) Seven [illegible]

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measu[illegible] after overnight fasting Both were expressed as mmol/h Fasting *serum gastrin* was determined by radioimmunoassay as earlier described in detail (24) The antibody used was No 2604 (18) which measures the four main gastrin components (gastrin 14 gas

shift in the distribution of immunoglobulin producing cells towards the type matching the serum M component In multiple myeloma usually more than 95% of the immunoglobulin producing cells produce the same immunoglobulin class (6 15) Though this figure is lower in BMG some overlapping exists and no clear distinction between the two conditions can be made (6 15) This also holds true for immunohistochemical examina ion By immunoperoxidase staining of bone marrow sections in multiple myeloma the light and heavy chain type can easily be achieved and corresponds in the vast majority of cases to the serum M component (5 13) Contrary to *Taylor et al* (13 14) we were able to identify the heavy and light chain type in multiple myeloma as well as in BMG by examination of immunoperoxidase stained tissue sections and in both conditions the result corresponded to the serum examination Though plasma cells producing immunoglobulin classes not matching the serum M component are generally more numerous in BMG than in multiple myeloma this is not always so and in our experience immunoperoxidase stained bone marrow sections offer no further information for making a distinction between BMG and multiple myeloma Identification of discrete marrow infiltrates from malignant lymphomas producing monoclonal immunoglobulin is facilitated by the immunoperoxidase technique illustrating the monoclonal cytoplasmic immunoglobulin

We wish to thank cytotechnologists *Dorthe Thuen* and *Preben Sandahl* for expert technical assistance in performing the enzymecytochemical and immunohistochemical reactions

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in Table 1 It is seen that eight weeks of treatment with cimetidine results in a significant increase in N_A (13%) and N_V (15%) and a decrease in $\bar{V}$ (11%) The latter is greater than the median value of CE before and during treatment No change was found in the value of V_V Beside alterations of G cell density the morphology as seen in the light microscope were unchanged before as well as during treatment with cimetidine Serum gastrin obtained in 26 pairs of patients was increased during treatment but the difference was insignificant

DISCUSSION

Elevated serum-gastrin caused by vagotomy or histamine H_2-receptor antagonists is probably induced by reduced gastric acid secretion (9 21) and must be due to a G-cell change either a hyperplasia and/or hyperfunction Earlier results after vagotomy are conflicting since in two series G-cell hyperplasia was found (5 8) whereas in another a decrease of G-cell density was reported (7) A difference in the sensivity and specificity of the antigastrin may be an explanation of the conflicting results as prerequiste to identification is a content of gastrin

Several authors have demonstrated ultrastructural changes during stimulation and secretion from the G-cells especially in the granulae (4 11 13 16 25)

This study shows that cimetidine treatment of duodenal ulcer patients results in a G-cell hyperplasia and that the volume of the individual cell decreases to such an extent that the volume density remains unchanged Assuming the antral mucosal volume remains the same both before and during treatment then the total G-cell volume will also remain unchanged *Witzel et al* (27) also found unchanged volume density during medication of rats with metiamide but they report no calculation of the number of G-cells

The G-cell hyperplasia after eight weeks of treatment with cimetidine and the elevated levels of serum gastrin even six weeks after withdrawal (6 10) may be a factor of importance with regard to the frequently rapid recurrence of an ulcer at times presenting as perforation after short term treatment (23 26) In contrast other authors (3) did not find a higher frequency of re ulceration after a short successfull course of cimetidine compared to a successful »course« of placebo It has also been demonstrated that the parietal cell sensitivity to pentagastrin remains unchanged after short term treatment with cimetidine (1)

Further studies ... ify an eventual autonomous function of the increased number of G cells after cimetidine treatment as a factor in recurrence of the ulcer

It is concluded that cimetidine treatment of duodenal ulcer patients results in antral gastrin cell hyperplasia without changes in the total G-cell volume and that this hyperplasia may be a factor of importance with regard to the rapid recurrence of many ulcers after cessation of the treatment

The anti gastrin serum No 2717 was kindly donated by Professor *J F Rehfeld* Institute of Biochemistry Aarhus University The authors wish to thank Professor *Daniel Andersen* Dept of Surgical Gastro-enterology Odense University Hospital for his advice and review of the manuscript The study was supported by grants from the *Danish Medical Research Council* (Journal No 512 5255 and 512 679)

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trin 17 gastrin 34 and component I) with almost equimolar potency (19) The concentrations were expressed in pg/ml *Fasting serum gastrin* was measured before and at the end of eight weeks of treatment

Tissue preparation Three biopsies were taken approximately 2 cm orally to the pyloric ring in connection with gastro duodenoscopy before and after eight weeks of treatment The biopsies were immediately fixed in 4% methanol free paraformaldehyde pH 7 4 and embedded in paraffin without any attempt at orientating the biopsies Three 5 μm thich sections were cut at a distance of 25 μm from each biopsy

Immunohistochemistry The G cells were identified as described earlier (14) by means of the indirect immuno peroxidase technique using anti gastrin serum No 2717 raised in rabbits against synthetic human gastrin I (18) which in radioimmunoassay acts as anti gastrin No 2604 although with a slightly increased cross reaction with cholecystokinin compared to antibody No 2604 (20) 3 amino 9 ethyl carbazol (Sigma Chemical Comp St Louis U S A) was used for the development The following controls were employed a) exclusion of the anti gastrin serum b) reaction between anti gastrin serum and gastrin (synthetic human gastrin I Calbiochem Bering Corp U S A) 10 μg per ml diluted anti serum before applying to the sections c) letting the anti gastrin serum react with cholecystokinin octapeptide (Calbiochem Bering Corp U S A) 15 μg per ml diluted anti serum before applying to the sections

Morphometry This was carried out as described earlier (15) where the error of measurement was characterized with regard to between and within observer variation as well as within patient variation and showed that acceptable levels of variation in endoscopic biopsies were reached when a minimum number of about 200 G cell profiles were counted The following variables were estimated 1) volume density (V_V) of G cells which relate the volume of G-cells to that of antrum mucosa volume 2) Numerical density on area (N_A) i e the number of G cell profiles per antral mucosal area 3) Numerical density (N_V) i e the number of G cells per antral mucosal volume and finally 4) Mean G cell volume ($\bar{V}$) The coefficient of error (CE) was determined for the variable with the greatest variation $\bar{V}$ in every count (15) The proportion between the long and short axis of the G cell profile was determined from randomly selected fields of vision using a Reichert projecting microscope to ensure that this proportion did not change during treatment with cimetidine

Section thickness This was controlled by a Tesatronic Electronetester (TESA S A Renens Schweiz)

Statistical methods The Wilcoxon test for paired data was used Differences were considered statistically significant when $P < 0\ 05$

RESULTS

The control staining a) and b) were negative In c) a 10% reduction of the G cell profiles was seen indicating some degree of cross reactivity between anti gastrin and cholecystokinin on the cellular plane In the morphometric analysis 30 pairs of patients fulfilled the minimum number of G-cell counts giving acceptable levels of error (15) only biopsies including muscularis mucosa were used for morphometry The median value of the proportion between the long and short axis of the G-cells was 1 63 (observed range 1 51 to 1 78) before treatment (n = 179) and 1 65 (observed range 1 49 to 1 99) during cimetidine treatment (n = 194) ($P > 0\ 1$) indicating the same proportion before and during treatment The median value of CE was 6% before (observed range 3 to 12%) and 6% during treatment (observed range 4 to 11%) The results of the morphometric analysis the fasting serum gastrin and acid secretion before treatment are seen

TABLE I *Measurements before and during Treatment with Cimetidine*

	Before treatment Median (observed range)		During treatment Median (observed range)		
Basal acid output (BAO) (mmol/h)	1 38	(0–9 3)			
Peak acid output (PAO) (mmol/h)	32 9	(10 9–67 9)			
Serum gastrin (pg/ml)	58	(27–165)	71	(39 155)	xx
Volume density of G cells (V_V) (% mucosal volume)	2 42	(1 42–3 21)	2 49	(1 55–4 37)	x
Number of G cells per area (N_A) (cells/mm²)	294	(152–436)	332	(195–593)	xxx
Numerical density of G cells (N_V) (cells/mm³)	23687	(11166–34727)	27149	(15492–50481)	xxx
Mean G cell volume ($\bar{V}$) (μm³)	1004	(790–1331)	896	(779–1052)	xxx

x $P > 0\ 10$ xx $P < 0\ 10$ xxx $P < 0\ 01$

n Table 1 It is seen that eight weeks of treatment with cimetidine results in a significant increase in N_A (13%) and N_V (15%) and a decrease in $\bar{V}$ 11%) The latter is greater than the median value of CE before and during treatment No change was found in the value of V_V Beside alterations of G cell density the morphology as seen in the light microscope were unchanged before as well as during treatment with cimetidine Serum gastrin obtained in 26 pairs of patients was increased during treatment but the difference was insignificant.

DISCUSSION

Elevated serum gastrin caused by vagotomy or histamine H_2-receptor antagonists is probably in h ced by ... hyperplasia was found (5 8) whereas in another a decrease of G-cell density was reported (7) A difference in the sensivity and specificity of the antigastrin may be an explanation of the conflicting results as prerequiste to identification is a content of gastrin

Several authors have demonstrated ultrastructural changes during stimulation and secretion from the G-cells especially in the granulae (4 11 13 16 25)

This study shows that cimetidine treatment of duodenal ulcer patients results in a G-cell hyperplasia and that the volume of the individual cell decreases to such an extent that the volume density remains unchanged Assuming the antral mucosal volume remains the same both before and during treatment then the total G-cell volume will also remain unchanged *Wu et al* (27) also found unchanged volume density during medication of rats with metiamide but they report no calculation of the number of G-cells

The G-cell hyperplasia after eight weeks of treatment with cimetidine and the elevated levels of serum gastrin even six weeks after withdrawal (6 10) may be a factor of importance with regard to the frequently rapid recurrence of an ulcer at times presenting as perforation after short term treatment (23 26) In contrast, other authors (3) did not find a higher frequency of re ulceration after a short successfull course of cimetidine compared to a successful »course« of placebo It has also been demonstrated that the parietal cell sensitivity to pentagastrin remains unchanged after short term treatment with cimetidine (1)

Further studies are needed to clarify an eventual autonomous function of the increased number of G cells after cimetidine treatment as a factor in recurrence of the ulcer

It is concluded that cimetidine treatment of duodenal ulcer patients results in antral gastrin cell hyperplasia without changes in the total G-cell volume and that this hyperplasia may be a factor of importance with regard to the rapid recurrence of many ulcers after cessation of the treatment

The anti gastrin serum No 2717 was kindly donated by Professor *J F Rehfeld* Institute of Biochemistry Aarhus University The authors wish to thank Professor *Daniel Andersen* Dept of Surgical Gastro-enterology Odense University Hospital for his advice and review of the manuscript The study was supported by grants from the *Danish Medical Research Council* (Journal No 512 5255 and 512 679)

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IN SITU IDENTIFICATION OF INFLAMMATORY CELLS IN MALIGNANT, NON-LYMPHOID HUMAN TUMOURS

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Svennevig J L *In situ* identification of inflammatory cells in malignant non lymphoid human tumours Acta path microbiol scand Sect A 88 387-395 1980

Inflammatory cells (the term is considered to include lymphocytes plasma cells macrophages mast cells and PMN) were identified and quantitated in sections from human carcinomas Two types of cellular infiltrates are described In the stroma surrounding the cancer tissue (peritumoural) and to a lesser degree also within the malignant tissue (intratumoural) lymphocytes plasma cells macrophages and mast cells dominated whilst numerous polymorph nucleated cells (PMN) and aggregates of macrophages characterized central tumour necroses The demonstration of both T lymphocytes and macrophages in and around the cancer tissue supports the view that a local immune reaction is initiated by the tumour and the consistent finding of IgG IgA and IgM plasma cells at the borders of the carcinomas indicates that plasma cells are also part of an immune response at the tumour site This view is further supported by the demonstration of an increased proportion of IgG containing plasma cells in the stroma of colon tumours compared with normal mucosa

Key words Inflammatory cells non lymphoid tumours

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ceived 13 v 80 Accepted 6 vi 80

Several reports have indicated that a cellular sponse may take place in and around malignant sues The nature of this host response has only en sparsely investigated and it is not proven hether human tumours possess new antigenic aracteristics which could explain the induction of local reaction

Many investigators have been able to demon rate a positive correlation between the degree of mphoreticular infiltration and prognosis (*Underood* 1974) By analyzing these cell infiltrates the pe of reaction taking place within human tumours lay be better understood Until now most studies ave dealth with the *in vitro* characterization of flammatory cells liberated from the neoplasms he aim of the present study was to identify the ifferent cell types infiltrating human malignant imours and to study the distribution of these cells ithin the tumours and in the peritumoural stroma n contact with the cancer tissue

MATERIAL AND METHODS

Non lymphoid tumours from 14 patients undergoing operation in Departments 2 and 3 Ullevål Hospital were examined The material included 5 adenocarcinomas of the colon 3 adenocarcinomas and 1 ductal carcinoma of the breast and 4 bronchial carcinomas (2 squamous cell carcinomas 1 adenocarcinoma and 1 poorly differentiated carcinoma)

Lymphocytes Lymphocytes were counted in 6 µm thick sec [illegible]

[illegible] 10 fields including the stroma tissue in contact with the cancer tissue (peritumoural)

T lymphocytes Fresh biopsies from all *tumours* were fixed in formalin sucrose overnight then in gum sucrose overnight and frozen sections were incubated for 22 hours with α naphthyl acetate (Sigma) The method was tested on normal lymph nodes and spleen where it gave distinct red brownish dots in the cytoplasm of the

lymphocytes in T dependent areas while cells in follicular areas were negative The ANAE method (*Knowles and Holck* 1978) was used to identify T cells in sections from all tumours and the number of T cells was expressed as percentage of all lymphocytes

B lymphocytes This population was included among esterase negative lymphocytes together with »null« lymphocytes No specific method for the demonstration of lymphocytes with membrane bound Ig was available when non lymphoid tissues were considered even when these cells could be easily demonstrated in cell suspensions from the same type of tumours (*Svennevig et al* 1978 1979)

Plasma cells IgG IgA and IgM containing cells were identified in and around the cancer tissue by using the peroxidase antiperoxidase method of *Taylor and Burns* (1974) on formalin fixed paraffin embedded material Six μm thick sections were mounted on gelatine chromalum treated coverslips deparaffinized (xylol) dehydrated (abs alcohol) and treated for 17 minutes with a 0 1% pronase (Boehringer Mannheim GmbH) solution Endogeneous peroxidase was blocked by placing the preparations in 3% hydrogen peroxide in methanol for 25 minutes The sections were then covered with heavy chain specific rabbit anti human IgG IgA and IgM and with kappa and lambda chain specific antisera (DAKO immunoglobulins A/S) thereafter with swine anti rabbit IgG (DAKO) and finally with soluble complex of horseradish peroxidase and rabbit anti horseradish peroxidase (DAKO) for 30 minutes respectively each step performed at room temperature and followed by a 15 minute wash in trisbuffer pH 7 6

The reaction was made visible with diaminobenzidin (Sigma) and hydrogen peroxide and the preparations were faintly counterstained with haematoxylin dehydrated cleared and mounted in canadabalsam

The specificity of the antisera was tested on different myelomas where they gave selective staining of cells containing one Ig class and on normal colon mucosa All preparations were further tested by changing the heavy chain specific antisera with rabbit Ig (DAKO) a[illegible] with phosphate buffered saline to ensure that no n[illegible] specific reaction had taken place

Both plasma cells and lymphocytes were counted [illegible] corresponding central and peripheral fields in paral[illegible] sections from each tumour and the number of t[illegible] different cell types was given as the average cells [illegible] field

Mast cells The 6 μm thick sections were deparaffiniz[illegible] rinsed in alcohol and stained for 10 (5–30) minutes w[illegible] pinacyanol erythrosinate (*Bensley* 1952) differentiated [illegible] abs alcohol rinsed in xylene and mounted in canadab[illegible] sam

This method lead to purple staining of mast cell[illegible] whereas both epithelial and muscle cells were sky bl[illegible] with bright blue nuclei

Macrophages A representative sector of each tumo[illegible] was fixed in formalin acetone overnight then in gu[illegible] sucrose overnight and frozen sections were incubate[illegible] for 22 hours with α naphthyl butyrate for demonstratio[illegible] of diffuse esterase activity (*Svennevig et al* 1979[illegible] Monocytes/macrophages were counted in 10 central an[illegible] peripheral fields selected at random

RESULTS

In all 14 tumours studied the stroma surrounding the malignant cells contained varying numbers o[illegible] inflammatory cells and these cell infiltrates wer[illegible] mainly composed of lymphocytes plasma cells macrophages some mast cells and PMN Th[illegible] majority of PMN were neutrophils except for colon carcinomas where a large portion of PMN were eosinophils There were no evident signs of tumour cell necrosis on the borders between the › host ‹ cells and the neoplastic cells All cell types were also found to infiltrate the cancer tissue however the intratumoural cell reaction was sparse compared

TABLE I *The Number of Inflammatory Cells/Microscopic Field (at Magnification 12 5 × 40) in and around 14 Human Carcinomas (Mean Value and Range)*

		Lymphocytes	Plasma cells	Macrophages	Mast cells	PMN
5 colon carcinomas	Intratumoural	4 (0 8–7)	1 4 (0 3)	9 (1–30)	0 1 (0–0 3)	4 (1–8)
	Peritumoural	35 (20–46)	36 (22–82)	26 (11–46)	4 5 (0 2–14)	10 (2–23)
	Adjacent mucosa	19 (5–35)	56 (29–77)	10 (1–19)	7 (1–16)	10 (2–14)
5 mammary carcinomas	Intratumoural	3 5 (0 6–9)	0 3 (0–0 6)	0 8 (0–2)	0 9 (0–2)	0 2 (0–0 5)
	Peritumoural	25 (2–60)	11 (3–17)	8 (1 5–20)	5 (0 4–7)	1 0 (0–1 6)
4 bronchial carcinomas	Intratumoural	8 (0 8–23)	5 6 (0 8–12)	10 (8–16)	0 1 (0–0 2)	2 (0 5–4)
	Peritumoural	76 (11–150)	53 (31–66)	17 (11–22)	4 (0 8–8)	2 5 (1–4)

ith the peritumoural stromal reaction (Table 1) The average number of inflammatory cells found in each tumour was reproducible by repeated counting

Central tumour necroses were frequently found and these areas were dominated by aggregates of PMN and macrophages Because of necrosis and cell debris it was not possible to quantitate the number of cells in these areas

There were some differences between the three tumour types studied All five colon carcinomas which belonged to Dukes stage C contained large numbers of lymphocytes plasma cells and macrophages (Fig 1) These cells were mainly found in the stroma between and around the neoplastic tissue (peritumoural) where lymphocytes often formed dense zones in close contact with the cancer tissue The strongest stromal reaction was seen in the superficial parts of the tumours while the cell infiltrates surrounding groups of cancer cells infiltrating musculature and pericolic fat tissue were sparse

Large macrophages often contained drops of lipid like material and macrophages were occasionally *found* diffusely distributed within the malignant tissue (Fig 1A)

Lymphocytes and plasma cells dominated in colon carcinomas On an average 33% of the intratumoural and 35% of the peritumoural lymphocytes were T-cells according to the ANAE staining pattern In some lymphocyte rich areas T cells dominated (Fig 1B)

Accumulations of IgG and IgA plasma cells were found close to the cords and nests of cancer cells (Fig 1C D) while *IgM* containing plasma cells were most often found as single cells in the stroma Only a few Ig-containing plasma cells of all three types were found to infiltrate the cancer tissue

There were no differences with regard to the total number of cells in the ›transitional‹ mucosa between tumour and normal intestinal wall and the tumour stroma but the tumour stroma was richer in lymphocytes whereas the number of IgA containing plasma cells was decreased The relative proportion of plasma cells containing IgG was therefore increased in the connective tissue of the tumours compared with the non malignant ›transitional‹ mucosa adjacent to the tumour and normal mucosa taken at least 8 cm proximally or distally form the tumour (Table 2)

A few single mast cells were located within the neoplastic tissue but the majority of these cells were also found in the peritumoural stroma The mast cells contained typical metachromatic granules which made the identification of the cells easy (Fig 1E) Although a significant accumulation of mast cells had taken place at the edges of the colon

TABLE 2 *The Relative Proportion of Plasma Cells with IgG IgA and IgM in Peritumoural Stroma Mucosa adjacent to Tumour and Normal Mucosa*

	IgG	IgA	IgM
Colon carcinomas	51	40	9
Adjacent mucosa	23	66	11
Normal mucosa	3	93	4
Mammary carcinomas	31	62	7
Bronchial carcinomas	41	36	23

carcinomas the highest number of mast cells was found in the transitional mucosa from the same cases

Only a few PMN were located within and around the cancer tissue However large ulcero necrotic areas of various sizes were present in all colon tumours These areas were dominated by numerous PMN and macrophages some lymphocytes and cell debris (Fig 1F) The necroses also contained a few IgG and IgA plasma cells

The average number of all cell types identified in and around the colon carcinomas is given in Table 1

Dense peritumoural cell infiltrates were also present in the four bronchial carcinomas Similar to the cell infiltrates found in colon tumours lymphocytes plasma cells and macrophages dominated except for necrotic areas with numerous PMN A relatively high number of plasma cells were present in these tumours and the relative proportion of IgG containing plasma cells was comparable with the proportion found in colon tumours (Table 2) Of the lymphocytes on an average 36% had T-cell markers

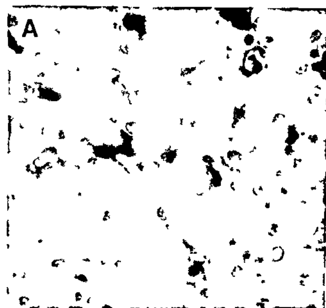
Although the morphology was less distinct with regard to the differentiation in a stromal and a neoplastic tissue the histological picture of the *bronchial tumours* gave the impression that an evident accumulation of mononuclear cells had taken place at the borders of the cancer tissue

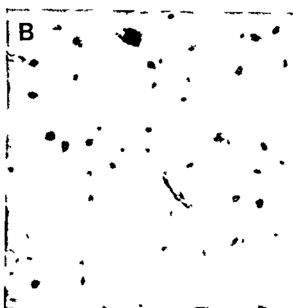
Mast cells heavily loaded with purple stained *granula* were numerous in the peritumoural stroma The cells were oval or spindle shaped and most cells were diffusely distributed in the stroma while some cells were found to line the cancer tissue and the stromal vessels They were only occasionally seen in necrotic areas of the tumours

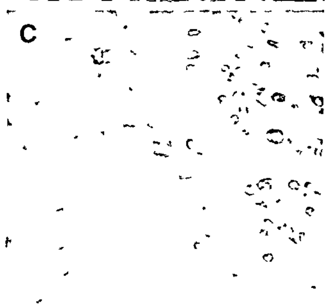
The mildest inflammatory cell reaction was found in mammary carcinomas In these tumours practically no central tumour necroses were present Lymphocytes plasma cel

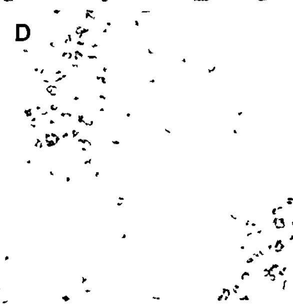
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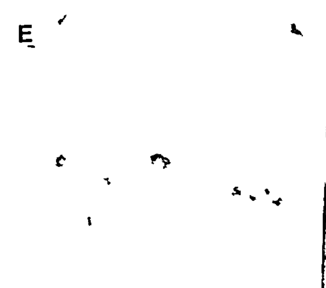
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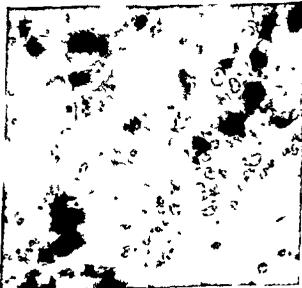

A


B


C


D


E



A Inflammatory cells in and around a colon nocarcinoma

B Intratumoural infiltration of macrophages and lymphocytes α naphthyl butyrate esterase staining 6 μm cryostat section × 500

C Peritumoural lymphocyte zone with ANAE positive T-cells 6 μm cryostat section × 1250

D IgG-containing plasma cells lining the cancer tissue (immunoperoxidase 6 μm paraffin section × 500

E Neoplastic tissue with groups of IgA-containing plasma cells and lymphocytes Technique as Fig C

F Mast cells lining the cancer tissue Pinacyanol staining 6 μm paraffin section × 500

F Intratumoural necrotic area with aggregates of macrophages and PMN Technique as Fig A

PMN A relatively constant proportion of lymphocytes both in (33%) and around the cancer tissue (38%) had T-cell markers Most macrophages were diffusely distributed in the connective tissue of the tumours

Of the plasma cells IgA-containing cells dominated followed by IgG and IgM In both mammary and colon tumours there was a slight dominance of lambda chain Ig whereas plasma cells with kappa chain Ig were most frequent in bronchial tumours

When the total number of inflammatory cells in the tumours was considered (Table 3) the intratumoural cell infiltration of mammary carcinomas expressed as cells/microscopic field was significantly weaker than the infiltration found in bronchial and colon carcinomas ($p < 0.05$) This was also the case when the intratumoural reaction was expressed as number of inflammatory cells/number of tumour cells in mammary carcinomas on an average 1% of the tumour cells were of an inflammatory nature compared to 6 and 9% respectively in colon and bronchial carcinomas (Table 3)

The stromal infiltration of inflammatory cells was 6–8 times stronger than the intratumoural infiltration for all three tumour types when the necrotic areas were not considered

DISCUSSION

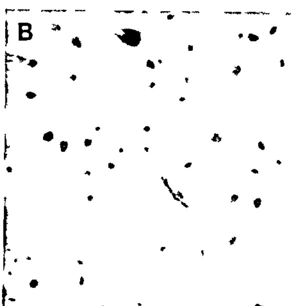
The present work represents an attempt to quantitate and characterize the inflammatory cells infiltrating and surrounding the malignant tissues in a series of human non lymphoid tumours By studying the inflammatory cells in corresponding microscopic fields by means of different staining techniques performed on serial tissue sections we were able to analyse the nature of the infiltrates and to compare the local cell reaction of different tumour types

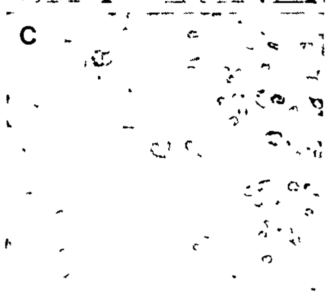
The infiltrates which are often termed »mononuclear cell infiltrates« or »round cell infiltrates« were composed mainly of lymphocytes plasma cells and macrophages but varying numbers of mast cells and PMN were also present Most cells were found in the stroma between and around the cords and nests of cancer cells (peritumoural) No evident signs of cell necrosis were present on the borders between host cells and neoplastic cells and a few inflammatory cells were diffusely distributed among the cancer cells Even electronmicroscopic studies have failed to demonstrate any cytotoxic effect of inflammatory cells close to neoplastic cells (*Carr and Underwood* 1974) However in some tumours central necroses were found and these areas were dominated by aggregates of PMN and macrophages While a marked lymphocyte infiltration may improve prognosis the presence of

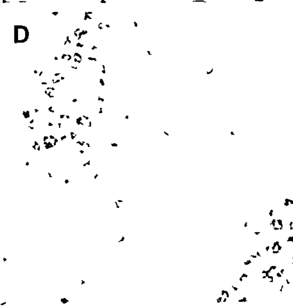
TABLE 3 *The Total Number of Inflammatory Cells in Areas mainly Composed of Neoplastic Cells and in the Peritumoural Stroma (Mean Value and Range)*

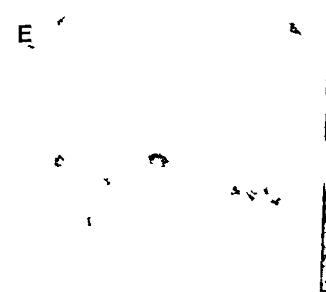
Tumour type	Cancer tissue		Peritumoural stroma
	Inflammatory cells	Cancer cells	Inflammatory cells
Colon carcinomas	19 (6–41)	301 (180–529)	112 (83–164)
Mammary carcinomas	6 (3–10)	497 (318–778)	50 (17–94)
Bronchial carcinomas	26 (9–51)	282 (101–356)	153 (59–206)

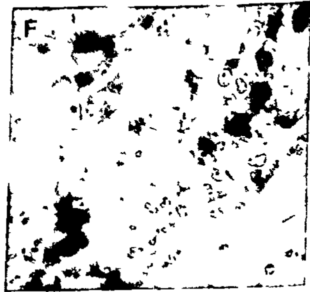

A


B


C


D


E


F

d Gillespie 1975) and human tumours (*Lauder et al* 1977)

The accumulation of mast cells in chronic inflammation and around carcinomas was described already by *Ehrlich* in 1865 (see *Selye* 1965) but the exact role of the mast cell reaction in malignancies is still not clear An increase in the number of mast cells has already been shown to take place prior to the development of experimental carcinomas (*Cramer and Simpson* 1944) and in carcinoma *in situ* *Dunn and Montgomery* 1957 *Graham and Graham* 1966) the mast cell reaction is thus suggested to be associated with a defensive process against cancer *Cramer and Simpson* 1944 *Fisher and Fisher* 1965 *Graham and Graham* 1966) However the release of granular material from tissue mast cells has been shown to promote tumour growth (*Sylven* 1945) while heparine binding substance may inhibit tumour growth (*Csaba et al* 1960)

The distribution pattern of the inflammatory cells in the present series of tumours was comparable to the type of reaction found in autochthonous rat transplants of low antigenic tumours where mononuclear cells tended to scatter around the tumour whereas high antigenic tumours were associated with massive immigration of mononuclear cells which extended throughout the tumour (*Kikuchi et al* 1976) A similar distribution of lymphocytes and macrophages has been described for growing murine tumours (*Wood and Gollahon* 1978)

The present study demonstrated that the »round cell« infiltrates surrounding and to a lesser degree infiltrating the malignant human tumours mainly consisted of lymphocytes (including T-cells) plasma cells and macrophages These cells formed the peri/intertumoural zones together with a few mast cells and PMN while PMN and macrophages dominated in central ulcero-necrotic areas The latter type of reaction may be secondary to the necrosis

On the basis of these findings and in accordance with some previous reports (*Burg and Braun Falco* 1972 *Elston* 1968 *Ioachim et al* 1976 *Takahashi* 1961) it seems difficult to speak of tumour inf

quite different mechanisms

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tumour necroses is considered to be an ominous prognostic sign (*Fisher et al* 1975) Except for these necroses which may be explained by insufficiency of blood supply in growing tumours the cells dominating the tumour infiltrates were mainly identical to the cells known to act as mediators of cellular and humoral immunity suggesting that a local host reaction to the neoplastic tissue may take place The present findings are in agreement with the results of our previous works demonstrating T and B lymphocytes macrophages and PMN in cell suspensions from different human non lymphoid tumours (*Svennevig et al* 1978 1979 *Svennevig and Svaar* 1979)

The significance of lymphocytic infiltrates in human malignant tumours has been discussed for many years Most but not all studies confirm the prognostic advantage of dense mononuclear cell infiltration (*Underwood* 1974)

We were able to demonstrate T lymphocytes in all tumours On an average 35% of the peritumoural and 33% of the intratumoural lymphocytes were T-cells which is in contrast to some previous studies using T-cell antisera (*Husby et al* 1976 *Schoorl et al* 1976) However our findings are in agreement with several works on lymphocytes liberated from human non lymphoid tumours reporting that most often less than half of the tumour lymphocytes were T-cells (*Havry and Totterman* 1978 *Hersh et al* 1975 *Svennevig et al* 1978 1979 *Vose et al* 1977 a b) The E rosette technique which is the most important marker for T lymphocytes in cell suspensions (*Jondal et al* 1972) may fail when applied on tissue sections (*Edelson et al* 1973 *Walker* 1976) and well characterized antisera against human T lymphocytes have until recently been difficult to obtain (*Braathen et al* 1979)

By isolating lymphocytes from disaggregated tumours we have previously found that 5–30% (mean 11%) of the lymphocytes had membrane bound immunoglobulin According to some reports B-cells may be identified in tissue sections by means of immunofluorescence or immuno peroxidase techniques however the term B-cell has also included cells with cytoplasmatic Ig (*Husby et al* 1976) We find it difficult to identify lymphocytes with membrane bound Ig safely in tissues other thar lymphoid even when the same technique may be used to identify Ig producing cells (plasma cells) in the same sections The identification of B lymphocytes by binding AEC rosettes to tissue sections (*Catalona et al* 1975 *Silveira et al* 1972 *Stingl et al* 1977) may be useful for lymphoid tissues or when groups of cells are studied but the technique is less suitable for the study of single cells

The role of plasma cell infiltration in and arou[nd] malignant tissues has been sparsely investigated [a]nd there are conflicting reports with regard to [the] content of plasma cells in human tumours *Gerst[l et] al* (1977) were able to demonstrate plasma cells [in] or near tumour tissue in only 6 out of 12 cases w[ith] bronchial squamous cell carcinomas while *Schoo[rl] et al* (1976) reported the finding of a small b[ut] constant number of plasma cells containing IgA a[nd] IgM in a series of breast carcinomas or occasionally were IgG producing cells found *Ro[g]num et al* (1979) found the number of Ig produci[ng] cells significantly increased in the non maligna[nt] stroma adjacent to colon carcinomas compared [to] normal mucosa whereas *Weis-Carrington et a[l]* (1976) and *Green et al* (1977) found the number [of] plasma cells markedly decreased In nasopharynge[al] carcinomas plasma cells of IgA (*Ho et al* 1978) an[d] IgG (*Konorza et al* 1979) have been reported [to] dominate

There are conflicting reports concerning th[e] correlation between the number of plasma cells an[d] the amount of tumour associated Ig (*Gerstl et a[l]* 1977 *Hurliman et al* 1976 *Roberts et al* 1973[)] and it is still not clear whether tumour bound Ig i[s] associated with an *in situ* expression of humora[l] immunity (*Witz* 1977) or whether it may act as a blocking factor of cellular cytotoxicity (*Romsdal and Cox* 1975)

The relative proportions of IgG IgA and IgM containing plasma cells in colon mucosa are shown in Table 2 The results are in agreement with the findings of *Rognum et al* (1979) who also found a relative increase of IgG containing plasma cells in the mucosa adjacent to the tumour

The present study demonstrated a further increase of the portion of IgG producing plasma cells in the tumour stroma and the demonstration of the relatively large portion of IgG-containing plasma cells in the tumour stroma contributes to the view that there exist stimulatory factors bypassing the secretory IgA immune system (*Rognum et al* 1979)

Macrophages were easily identified in the tissue sections by demonstration of their diffuse esterase activity Other authors have used the Fc receptor (EA rosette binding to tissue sections) as a marker for macrophages (*Catalona et al* 1975 *Roubin et al* 1975 *Stingl et al* 1977) These results may be difficult to interpret as other cell types in human tumours may also carry Fc receptors (*Tonder et al* 1974 *Vose et al* 1977 a b) Although the significance of macrophages in malignant tumours is still under discussion some recent reports emphasize their prognostic significance for both experimental (*Eccles and Alexander* 1974 *Wood*

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ULTRASTRUCTURE OF THE TAIL STUMP SPERM DEFECT IN THE BULL

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Blom E & Birch Andersen A Ultrastructure of the tail stump sperm defect in the bull Acta path microbiol scand Sect A 88 397–405 1980

A characteristic major sperm defect, with about 60% of the sperm cells showing a rudimentary tail stump was found in two sterile Danish bulls of imported origin The condition seldom occurs but seems to be widespread and has been observed in several breeds of cattle and in different animal species including man Electron microscopy of ejaculated sperm and testicular tissue from one of the bulls has shown that a dysfunction of the centriolar apparatus during spermiogenesis is likely to be the cause of the defect In ejaculated defective sperm the proximal centriole is often found connected with the normally transitory organelle the centriolar adjunct In the defective spermids the distal centriole has lost the faculty to form axonemal fibers so that no tail formation can take place

Key words Sperm defect tail stump sterility in bulls spermiogenesis distal centriole »dense granules« centriolar adjunct

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In 1964 *Coubrough and Barker* (9 10) described a characteristic sperm defect found in the semen of three Canadian bulls in which the majority of the sperms showed a rudimentary tail stump while the morphology of the sperm heads was normal The bulls were of Ayrshire Shorthorn and Holstein breeds respectively Eleven years later a similar case was found in a sterile Danish Friesian bull *G* the dam of which had been inseminated with imported American semen (6)

Recently another Danish case was found in an imported purebred Hereford bull This bull was also reported totally sterile For methods used for the evaluation of sperm morphology see ref 5

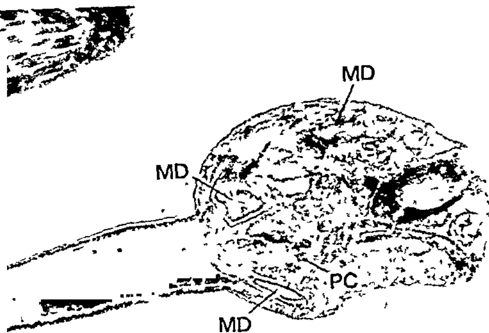
After publication of the first Danish case (6) we were informed of a German case in a Friesian bull (*G H Rieck* pers comm) a Swedish case in an SRB bull (*I Settergren* pers comm) and an Australian case in a beef bull (*R L Peet* pers comm) In Brazil a similar defect has been demonstrated in the semen of two inbred sterile Zebu bulls (17)

This particular sperm defect has also been found in the rabbit (14) the stallion and the dog (3) In mice an arrest in the development of the sperm tail has been demonstrated in a new mutant the »hop sterile« strain (13) This defect may be identical with the tail stump defect found in the other mammals mentioned In two infertile Italian men a similar »short tail« sperm defect has been reported (2)

MATERIALS AND METHODS

For electron microscopy an ejaculate was collected when the bull *G* was 21 months old The semen quality was very poor (Figs 1 & 2) 60% of the cells showed the tail stump defect the sperm concentration was low 250 mill /ml and only 10% of the cells were motile Samples were prefixed shortly after ejaculation by admixture of 7 ml of a solution of 0 1% glutaraldehyde in 3% sodium citrate to 2 ml of the ejaculate The

...um citrate) Blocks (about 1mm³) with clusters of



3

SC
DC
PC
AD
MD

4



Figures 1 and 2 Photomicrographs of India ink smears from ejaculates of bull *G* More than half of the sperms hav rudimentary tail stumps 450× and 1130× (From ref 6)

cells were cut after solidification of the agar and fixation was continued overnight at 4 °C in 3% glutaraldehyde in 3% sodium citrate After a brief wash in 0 1 M barbiturate buffer pH 7 3 the blocks were fixed for 2 hours at room temperature in 1% osmium tetroxide buffered with barbiturate pH 7 3 *En bloc* treatment for 1 hour with 2% uranyl acetate in the same buffer was used before dehydration through a graded ethanol series Finally the blocks were embedded in Vestopal W after propylene oxide treatment

Three months after the first ejaculates were obtained unilateral castration was done (left testis weight 400 g) Small blocks of tissue from the testis and cauda epididymidis were excised and prefixed within 10 minutes after removal in 6% glutaraldehyde in 0 1 M cacodylate buffer pH 7 2 After one hour the blocks were subdivided into pieces of 1 2 mm³ and transferred to fresh 6% glutaraldehyde for 5 hours of continued fixation The tissue blocks were stored overnight at 4 °C in 0 2 M sucrose in 0 1 M cacodylate buffer pH 7 2 before they were postfixed for 2 hours at room temperature in 1% osmium tetroxide in 0 1 M barbiturate buffer pH 7 3 containing 4 5% sucrose Finally treatment with uranyl acetate dehydration and embedding was carried out as described for the ejaculated cells Sections were cut with glass knives on an LKB ultrotome III microtome stained with magnesium uranyl acetate (12) and lead citrate (16) and examined with a Philips EM 300 electron microscope operated at 60 kV

After another five months the bull was slaughtered and tissue from the remaining right testis (weight 410 g) was studied after preparation in the same way as the material from the left testis

Testis tissue from two normal young bulls one slaughtered before and one after bull *G* was fixed and prepared in the same way and used as control material

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Figures 3–9 are electron micrographs of sections of ejaculated sperm cells and spermids from testis of bull *G* or from normal control bulls Sections poststained with uranyl and lead salts The bar on each micrograph represents 0 5 μm The following abbreviations are used throughout ad – centriolar adjunct an – annulus ax = axonemal fibers cb = chromatoid body dc – distal centriole gt – tail guide tube ma manchette md – membranous debris pc proximal centriole sc – segmented column

Figure 3 Ejaculated cell bull *G* Note the proximal centriole and the membranous debris in the tail stump region 45 000×

Figure 4 Ejaculated cell bull *G* The centriolar adjunct is present on the proximal centriole The distal centriole is seen distally to the segmented column 45 000×

5
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6
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RESULTS

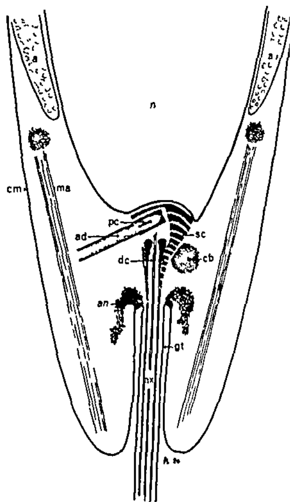
Electron microscopy of sections of *ejaculated sperms* from bull *G* (Fig 3) clearly demonstrated the contents of the droplet-like rounded body surrounding the implantation region of most of the »tailless« heads Frequently the normally transitory centriolar adjunct persisted and was attached to the proximal centriole (Fig 4) Up to this time the centriolar adjunct has never been observed in ejaculated sperm from bulls In some cases where the tail stump was cut rather peripherally it contained only an accumulation of different types of vesicles and membranous debris Frequently a mixture of remnants of the capitulum, the segmented columns, and/or dispersed mitochondria together with membranous debris was found to be enclosed by the cell membrane of the tail stump (Fig 4)

In sections of sperm cells from the *cauda epididymidis* the findings were similar, but the individual tail stumps appeared somewhat larger, as if their contents were not as condensed as demonstrated in the ejaculated cells

The different phases of spermiogenesis were studied in normal as well as in abnormal testis tissue in order to determine at what stage the pathological material showed deviations from the normal development of spermids thus attempting to elucidate the genesis of the tail stump defect

Through the early phases of spermiogenesis there were no differences between normal and pathological cells However from the acrosome phase when the transitory organelle the manchette was present (Fig 5) differences were noted In the bull this »manchette phase« as we prefer to call it most probably includes the stages A_2 and A_3 described in a study on spermiogenesis in the blue fox (1) To our knowledge a similar ultrastructural differentiation in the bull has not been established

In our normal material the implantation region of a spermid in the »manchette phase« contains the following organelles (Fig 6 and Text Fig 1) The proximal centriole with its transitory adjunct is situated distal to the basal plate The so called capitulum further contains the segmented column(s) with its implantation plate and the elongated distal centriole situated within the distal parts of the segmented columns A transitory chromatoid body



Text Fig 1 Schematic drawing of the implantation region from a bull spermid in the manchette phase Note the transitory organelles and the tail guide tube (see text) *a* = acrosome *ad* = centriolar adjunct *an* = annulus and accumulations of granular material *ax* = axonemal fibers *cb* = chromatoid body *dc* = distal centriole *gt* = tail guide tube *ma* = manchette *n* = nucleus *pc* = proximal centriole *sc* = segmented column – *Lars Blom* fec *Erik Blom* dir

is usually present on one or both sides of the capitulum at the level of the distal centriole In properly oriented sections the annulus is seen as two triangular dense bodies at the distal end of the segmented column The annulus is at the same time situated at the proximal end of the cell membrane invagination which has at this stage been formed around the tail primordium This configuration has

Figure 5 Testis normal bull The implantation region at the manchette stage Note the presence of distal centriole axoneme tail guide [illegible] manchette and a chromatoid body 45 000 ×

Figure 6 Testis nc [illegible]
proximal centriole [illegible]
segmented column are also present 45 000 ×

7

DC

GT

MA

MA

8

CB

GT

AN

DC

PC

GT

AD

been described as the »tail guide tube« (9) »the cytoplasmic invagination« (15) or the »cytoplasmic canal« (1)

In a favourably oriented section (close to the median axis of the tail) the axoneme can be followed from its base at the distal centriole and the annulus, and as far as the section and the field of view will allow (Figs 5 and 6) The number of axonemal fibers visible depends on the level and the thickness of the section

By light microscopy it was impossible to demonstrate typical pathological changes in the testis tissue from bull *G* However, the ultrastructure of the implantation region did show a pronounced difference between normal and abnormal spermiogenesis, especially from the »manchette phase« and further on *Many spermids in the* manchette phase were studied both in sagittal (Fig 7) and in horizontal sections (Figs 8 and 9) In the most successful horizontal sections all the previously mentioned more or less transient organelles the manchette (Fig 9) the centriolar adjunct, the chromatoid body and the distal centriole, could be seen in the section At this stage the detailed morphology within the neck region seemed to be almost identical with that found for normal bulls, but one important difference was noted In the typical abnormal spermid there was no axoneme formation *The »tail« simply terminated as a short* »stump« at the end of the distal centriole and no *axonemal fibers were present within the tail guide* tube which contained only cytoplasmic matrix and *some ribosomes (Figs 8 and 9) Sagittal sections of* three spermids in the manchette stage one of them with an *empty* tail guide tube, are shown in Fig 7

In addition to this main type of abnormality another characteristically abnormal detail was occasionally demonstrated in cells at the later stages of spermiogenesis and in ejaculated sperm from the bull *G* Clusters of 300–500 Å dense granules resembling those described earlier (7) were often seen in association with disorganized accumulations of mitochondria This type of arrangement was usually present in the peripheral cytoplasm of abnormal spermids or at the periphery of the cytoplasmic droplet of the »tailless« sperm cells

DISCUSSION

The tail stump defect in the bull is a characteri *sterilizing defect which affects only the sperm* While the ordinary histological picture of the te tissue from bull *G seems normal, the ultrastructu* studies have shown that spermiogenesis is nevert less seriously affected *The genesis of the defec* linked mainly to the function of the distal centri in early spermiogenesis i e in the *formation* of axonemal complex

In recent literature on the ultrastructure mammalian sperm very little information is ava able on the distal centriole It is an organelle whi loses its function during early spermiogenesis a in the mature sperm it is either completely absent *rudimentary, it is, in other words a »parti* transitory organelle In the bull sperm the so-calli »dense rods« have been identified as rudiments the distal centriole (4) According to other report however, the distal centriole does not disappe completely, but persists in a modified form as a so called »transitional connecting centriole« (18)

The following description has been given of th function of the distal centriole (8) »The earlie events in formation of the tail consist of migratio of the centrioles to cell surface in the postnuclea region and polymerization of microtubule protei on the template provided by *the distal centriole A* typical 9 + 2 axoneme is formed and the simpl *flagellum elongates by accretion of microtubul* subunits to its distal end« This early stage in the tai *formation is illustrated by Fawcett et al* (11) in thei Fig 7, where the tail primordium and the centriolai *complex* still are situated far from the nucleus

In the present study it was observed that ejaculated sperm with the tail stump defect could show retention of a typical transitory organelle such as the centriolar adjunct As far as we know the presence of the centriolar adjunct has not been observed previously in bull ejaculates However in normal human ejaculated sperm the presence of the centriolar adjunct is considered a sign of relative immaturity (18) It is therefore tempting to *suggest* that in the abnormal sperm cells of the present material the *normal function of the proximal* centriole is also affected

The dense granules found in our sections in the peripheral cytoplasm of abnormal spermids and in

Figure 7 Testis bull *G* *Three* spermids in manchette stage Note the distal centriole and empty tail guide tube in upper cell (see discussion) 8 000 ×

Figure 8 Testis bull *G Spermid in* manchette stage The proximal centriole and its adjunct is present together with the chromatoid body The distal *centriole ends in an empty tail guide tube* no axoneme is formed but the annulus is present 45 000 ×


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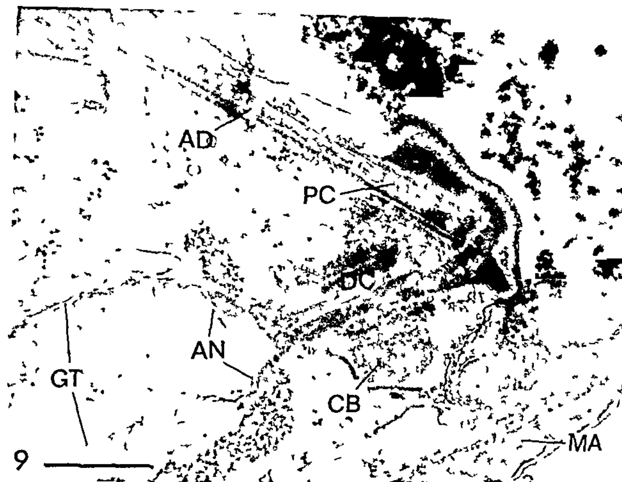


Figure 9 Testis bull *G* Spermid in manchette stage The proximal centriole is seen with its adjunct A chromatoid body and part of the manchette is also present Note the distal centriole the *empty tail guide tube and the annulus* 45 000 ×

sperm cells morphologically resemble those demonstrated in the pseudodroplet defect in the bull (7) However in the case of the tail stump defect in bull *G* the axonemal fibers are lacking in the defective spermids and this may have reference to the unorganized accumulation of clusters of mitochondria and dense granules At present no explanation can be given as to the origin of the dense granules neither in the pseudodroplet defect nor in the tail stump defect

It is peculiar that the tail guide tube appears to develop even if no axonemal fibers are formed (Figs 8 and 9) Normally this canal is present at a very early stage in spermiogenesis (1) but how it can possibly develop without a tail primordium is difficult to understand

Some doubt could probably be raised about the missing axonemes in our *illustrations (Figs 8 and* 9) of the abnormal spermids If a tail primordium happens to be cut peripherally in a longitudinal section the tail guide tube will most probably appear *empty However* with one exception our illustrations are from sections close to the median axis of the neck because the annulus appears as two well separated dense triangles Therefore if present some of the axonemal fibers should have been evident in the sections The one exception is the *sagittal section shown in Fig 7 where the annulus* seems to be hit tangentially and where the emptiness of the tail guide tube may therefore be doubtful

In summary the tail stump defect may be traced back to some defect(s) at an early stage of spermiogenesis which seems to originate in abnormal conditions in the centriolar complex causing *1)* a retention of the adjunct of the proximal centriole in ejaculated sperm cells and *2)* a complete or partial inhibition of the formation of the axoneme by the distal centriole

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Acta path microbiol scand Sect A 88 407–413 1980

LATE EFFECTS OF COLCHICINE ON THE BURSA OF FABRICIUS AFTER NEONATAL APPLICATION ON THE ANAL LIPS OF CHICKENS

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Romppanen T & Sorvari T E Late effects of colchicine on the bursa of Fabricius after neonatal application on the anal lips of chickens Acta path microbiol scand Sect A 88 407–413 1980

One week after the application of colchicine on the anal lips of chickens the bursa of Fabricius was devoid of lymphoid cells and contained only a few epithelial buds all of which were later repopulated with lymphoid cells to form mature lymphoid follicles After the intraperitoneal injection of cyclophosphamide numerous epithelial buds were found in the bursa and at the age of six weeks only a few of them were populated with lymphoid cells On week 6 the bursal weights of the chickens treated with colchicine were only about 10% of the normal and about 80% of the bursal weights of the cyclophosphamide treated chickens The colchicine treatment affected the morphology of the spleen less than the treatment with cyclophosphamide It is concluded that this new model of chemical bursectomy differs distinctly from the cyclophosphamide model and therefore serves as a new tool in studying bursal function in chicken

Key words Bursa of Fabricius chemical bursectomy colchicine

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To study the effects of various tissue components of the bursal microenvironment on the maturation of B cell lineage it is necessary to selectively destroy bursal lymphocytes For this purpose various cell poisons have been applied on the anal lips of the chickens Recently we described a new model of chemical bursectomy where bursal lymphoid cells were selectively destroyed together with the cells of the follicle associated epithelium by applying colchicine solution on the anal lips of chickens (12) During an observation period of one week no obvious direct effects of colchicine on thymus spleen or caecal tonsils were seen Therefore this model of chemical bursectomy seems to differ from the cyclophosphamide model where direct effects on the lymphoid tissues of the spleen caecal tonsils and thymus has been reported (5–7 9–11 13–18)

In this report late (1–10 weeks) effects of colchicine on the chicken bursa of Fabricius spleen

MATERIALS AND METHODS

Laboratory animals Newly hatched White Leghorn chickens were received from a local hatchery The chickens were given commercial chicken food and water *ad l b tum*

In this work two groups of con

was given on the day of hatching

TABLE 1 *Body Bursal and Spleen Weights (Mean ± S D)*

	Untreated	Treatment with colchicine	Treatment with cyclo phosphamide
Body weight (g)			
1 week	54 2 ± 5 2 (20)	57 8 ± 5 7 (25)***	42 1 ± 6 0 (10)***
2 weeks	86 3 ± 14 1 (5)	83 0 ± 9 4 (5)	
4 weeks	215 5 ± 24 9 (4)	213 4 ± 31 4 (7)	187 4 ± 22 3 (5)
6 weeks	433 0 ± 5 9 (8)	**362 2 ± 51 7 (9)*	263 3 ± 103 8 (9)***
10 weeks	958 5 ± 121 1 (10)	877 1 ± 126 1 (18)	
Bursal weight (mg/100 g b w)			
1 week	252 3 ± 55 1 (20)	***50 6 ± 18 6 (25)***	93 8 ± 18 8 (10)***
2 weeks	354 6 ± 59 8 (5)	***49 7 ± 28 2 (5)	
4 weeks	747 1 ± 124 0 (4)	***41 6 ± 23 5 (7)**	89 8 ± 24 8 (5)***
6 weeks	592 9 ± 138 9 (8)	***53 7 ± 24 7 (9)	67 1 ± 11 4 (9)***
10 weeks	411 9 ± 101 8 (10)	***20 5 ± 10 1 (18)	
Spleen weight (mg/100 g b w)			
1 week	112 1 ± 37 7 (20)	* 89 0 ± 19 8 (25)***	47 6 ± 20 9 (10)***
2 weeks	163 2 ± 19 7 (5)	134 3 ± 44 8 (5)	
6 weeks	270 5 ± 87 0 (8)	203 2 ± 65 7 (9)	213 4 ± 58 8 (9)
10 weeks	244 3 ± 60 2 (10)	235 6 ± 32 3 (18)	

The numbers in brackets indicate the number of chickens in each group
Marks beneath the values of the colchicine group indicate significant differences when compared to the untre controls (on the left) or to the cyclophosphamine group (on the right) Marks beneath the values of cyclophosphamide group indicate significant differences in a comparison to the controls * P≦0 05 ** P≦0 *** P≦0 001

The number of chickens in different experimental groups is presented in Table 1

Tissue sampling The chickens were killed 1 2 4 6 and 10 weeks after hatching with an overdose of ethyl ether The chickens their bursae and spleens were weighed Histological samples were taken from the transversal midline of the bursa spleen and caecal tonsils fixed overnight in 4 % neutral buffered formaldehyde and embedded in paraffin Tissue sections six μ thick were stained with Hematoxylin Eosin van Gieson Hematoxylin and methyl green and pyronin methods

Statistics Student s t test was employed in comparison of the means

RESULTS

Body bursal and spleen weights The body bur and spleen weights are presented in Table 1

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TABLE 2 *The Occurrence of Epithelial Buds and Lymphoid Follicles in the Bursa after Colchicine Treatment*

Age of chickens	Total number of bursae	No buds no follicles n[1]	No buds no follicles %[2]	Buds present no follicles n	Buds present no follicles %	No buds follicles present n	No buds follicles present %	Both buds and follicles n	Both buds and follicles %
1	25	7	28	15	60	1	4	2	8
2	5	2	40	2	40	0	0	1	20
4	7	2	29	0	0	0	0	5	71
6	9	0	0	0	0	1	11	8	89
10	18	0	0	0	0	16	89	2	11

1) Number of bursae
2) Percentage of total number of bursae



Fig 1 Bursa of a one week-old chicken treated with colchicine No lymphoid follicles present Small epithelial buds are indicated by arrows (H&E × 100)

Fig 2 Detail from figure 1 An epithelial bud with rudimentary cortical zone is seen The open arrow points to the follicle associated epithelium The closed arrows show the interfollicular surface epithelium (H&E × 400)

Fig 3 Bursa of a two week-old chicken treated with colchicine A few follicles (F) and epithelial buds (open arrows) are seen The closed arrow points to a cluster of small lymphocytes around a venule in the bursal stroma (H&E × 100)

Fig 4 Bursa of a two-week-old control chicken F = lymphoid follicles open arrows = follicle associated epithelium closed arrows = interfollicular surface epithelium (H&E × 100)

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		n[1)]	% [2)]	n	%	n	%	n	%
1	25	7	28	15	60	1	4	2	8
2	5	2	40	2	40	0	0	1	20
4	7	2	29	0	0	0	0	5	71
6	9	0	0	0	0	1	11	8	89
10	18	0	0	0	0	16	89	2	11

1) Number of bursae
2) Percentage of total number of bursae


5


6


7

TABEL 3 *The Number (Mean ± SD) of Lymphoid Follicles and Epithelial Buds in a Cross Bursal Section*

Age of chickens (weeks)	Epithelial buds			Lymphoid follicles		
	Untreated	Colchicine	Cyclophosphamide	Untreated	Colchicine	Cyclophosphamide
1	0 (3)	13 ± 28 (25)	627 ± 77 (5)	487 ± 3 (3)	7 ± 26 (25)[1]	0 (10)
2	0 (3)	27 ± 39 (5)		483 ± 64 (3)	10 ± 22 (5)	
4	0 (3)	54 ± 96 (7)	305 ± 70 (5)	502 ± 71 (3)	6 ± 13 (7)	3 ± 3 (5)
6	0 (3)	30 ± 29 (9)	417 ± 97 (9)	652 ± 68 (3)	30 ± 30 (9)	7 ± 9 (9)
10	0 (3)	0,4 ± 1,4 (18)		592 ± 83 (3)	16 ± 22 (18)	

1) In 22 cases 0, remaining 3 cases 7,90 and 96
The numbers in brackets indicate the number of animals in each group

with colchicine were compared with those of the untreated controls, a significant difference was noticed only on week 6 The body weights of the cyclophosphamide group were significantly lower at weeks 1 and 6, when compared with the other two groups

In every age group, the bursae of the chickens treated with colchicine were remarkably lighter than those of the untreated controls The effect of colchicine on the bursal weight appeared to exceed that of cyclophosphamide

In the colchicine group, the spleens were slightly lighter than those of the untreated control animals, but only on week 1 was this difference significant On week 1, the chickens treated with cyclophosphamide had spleens which were markedly lighter than in the other two groups

Histology of the bursa At the age of 1 week 28 % of the bursae in the colchicine group (Table 2) were totally devoid of both lymphoid follicles and epithelial buds, consisting only of fibrous stroma and interfollicular surface epithelium In 60 % of the bursae (Table 2) scattered epithelial buds (mean 13 per section, Table 3) with follicle-associated epithelium were found in the relatively increased bursal stroma (Figures 1 and 2) Practically no lymphoid follicles were observed in the bursae of the colchicine groups whereas in the bursae of the untreated animals they were abundant (mean 487, Table 3) The bursae in the cyclophosphamide group contained numerous epithelial buds thus differing distinctly from the bursae of the colchicine-treated chickens (Table 3)

At the ages of 2, 4 and 6 weeks the number of bursae containing lymphoid follicles increased in the colchicine group (Table 2), indicating a spontaneous lymphoid repopulation The number of these lymphoid follicles, however, was rather small when compared to the controls (Table 3, Figures 3 and 4) At this time, follicles in different stages of repopulation were seen in the bursae of the colchicine group In most follicles the repopulation seemed to begin in the medulla (Figure 5), although cortical accumulation of lymphoid cells was sometimes observed, too Some lymphoid cells around the buds in the stroma were also observed (Figure 5) In the bursae of the cyclophosphamide-treated chickens numerous epithelial buds were seen, but a repopulated lymphoid follicle only occasionally (Table 3) thus differing from the bursae of the colchicine-treated animals

At the age of 10 weeks, the bursae of the colchicine-treated chickens contained lymphoid follicles, but practically no epithelial buds (Table 2, Figure 6) The number of the lymphoid follicles remained low, however (Table 3) Numerous lymphoid cells were found diffusely in the stroma as well as aggregated in clusters beneath the interfollicular surface epithelium (Figure 7)

Histology of the spleen and caecal tonsils After the application of colchicine, the amount of lymphoid cells around the Schweigger-Seidel sheats seemed to be smaller when compared with the untreated controls, but greater than in the cyclophosphamide group On week 6, the number of

Fig 5 Bursa of a four week-old chicken treated with colchicine The medulla of an epithelial bud is populated with pyroninophilic cells (F) whereas the cortical zone is poorly developed Note the epithelial buds (B) with few scattered pyroninophilic cells in the medulla and the cortex (Methyl green and pyronin × 300)

Fig 6 Bursa of a ten week old chicken treated with colchicine Mature lymphoid follicles (F) are present but no epithelial buds are observed (H&E, × 200)

Fig 7 Bursa of a ten-week-old chicken treated with colchicine Lymphoid cells are scattered in the stoma subepithelially or aggregated as a follicle like structure (asterix) (H&E, × 400)

which is in accordance with earlier studies (10 14) and they were more pronounced than those in the colchicine model It is possible that after the treatment with cyclophosphamide there are not enough suitable lymphoid cells in the chickens to repopulate the bursal epithelial buds unless the chickens are transplanted with bursal cells (14–18)

According to our earlier (12) and present studies colchicine treatment induces a rather selective injury in the bursa affecting the lymphoid cells and the follicular epithelium This model of chemical bursectomy differs markedly from other models of bursectomy as discussed previously (12) and in this paper and apparently serves as a new tool in studying the development of the B cell lineage in the chicken bursa

The technical assistance of Mrs *Pirjo Kortevuori* Mrs *Irma Vaananen* and Mrs *Raija Pitkanen* is gratefully acknowledged

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TABEL 4 *The Number of Germinal Centers (Mean ± SD) in a Cross Spleen Section*

Age of chickens (weeks)	Number of germinal centers		
	Untreated	Colchicine	Cyclophosphamide
1	0 (20)	0 (25)	0 (10)
2	0 6 ± 1 3 (5)	0 (5)	
6	8 3 ± 6 3 (8)	2 6 ± 3 4 (9)[1)]	0 1 ± 0 9 (9)[2)]
10	7 1 ± 9 3 (10)	5 1 ± 8 9 (18)[3)]	

1) $P \leqq 0\ 05$ when compared to the controls
2) $P \leqq 0\ 01$ when compared to the controls $P \leqq 0\ 05$ when compared to the colchicine group
3) No significant difference in a comparison to the controls
The numbers in brackets indicate the number of animals in each group

germinal centers in a spleen section in the colchicine group was smaller than in untreated controls whereas on week 10 the difference was minimal (Table 4) The cyclophosphamide group distinctly differed from the other two groups because practically no germinal centers were found on week 6

Caecal tonsils were examined on week 10 In the colchicine treated chickens the amount of germinal centers and lymphoid cells in the stroma was smaller than in untreated controls The caecal tonsils of the cyclophosphamide treated chickens were not investigated in this study

DISCUSSION

After treatment with colchicine the bursa contained only a few epithelial buds while after an intraperitoneal injection of cyclophosphamide the number of buds i the bursa approximately equalled the number of follicles in the bursae of untreated control animals During the observation period all of the few epithelial buds in the colchicine treated bursae were populated by lymphoid cells to form mature histologically normal appearing lymphoid follicles The number of lymphoid follicles on week 10 however, was only about 3 % of that found in the bursal sections of the untreated control animals

The repopulation of each single epithelial bud by lymphoid cells seemed to take place independently because lymphoid follicles were found in different stages of repopulation This repopulation resembled either embryonal development of the bursal follicles (1 2) or states found in the bursae of the cyclophosphamide treated chickens spontaneously (5 7) or following transplantation with bursal lymphoid cells (14–18) The origin of the repopulating lymphoid cells remains unclear, (1) they can be descendants of lymphoid cells which survived t initial necrosis caused by colchicine or (2) they c be peripheralized bursal lymphocytes which ha returned to bursa or (3) they are lymphocytes fro a non bursal site of B cell development (8)

In the colchicine model no »acute« direct effec on the spleen or caecal tonsils has been demonstr ted (12) However, later the spleen weight th amount of lymphoid cells in the periellipsoid are and the number of germinal centers in the spleen a

secondary due to total depletion of the lympho cells in the bursa On week 10 increased weight and normalized morphology of the spleens indicate their partial recovery This injury recovery cyclus ir the bursa also partially reflected in the spleen ir our present model bears some resemblance to that caused by intramuscular testosterone propionate injection (4)

The chickens which received intraperitoneal injections of cyclophosphamide did not gain as much weight as those treated with colchicine a fact apparently indicating a more serious systemic effect Besides the total loss of lymphoid cells no other apparent damage was observed histologically in the bursae of the cyclophosphamide treated chickens Cyclophosphamide did not destroy epithelial buds As reported in earlier studies (6 7) in cyclophosphamide treated chickens only a part of numerous epithelial buds are spontaneously populated with lymphoid cells on week 10 while in the present study all of the few buds in the colchicine group were populated Thus the cyclophosphamide and the colchicine models also differ in their type of spontaneous lymphoid repopulation of the bursa which is less active in the former The effects of cyclophosphamide on the spleen were evident

which is in accordance with earlier studies (10 14) and they were more pronounced than those in the colchicine model It is possible that after the treatment with cyclophosphamide there are not enough suitable lymphoid cells in the chickens to repopulate the bursal epithelial buds unless the chickens are transplanted with bursal cells (14–18)

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1) P ≦ 0,05 when compared to the controls
2) P ≦ 0,01 when compared to the controls, P ≦ 0 05 when compared to the colchicine group
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The numbers in brackets indicate the number of animals in each group

germinal centers in a spleen section in the colchicine group was smaller than in untreated controls, whereas on week 10, the difference was minimal (Table 4) The cyclophosphamide group distinctly differed from the other two groups, because, practically no germinal centers were found on week 6

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DISCUSSION

After treatment with colchicine the bursa contained only a few epithelial buds, while after an intraperitoneal injection of cyclophosphamide the number of buds i the bursa approximately equalled the number of follicles in the bursae of untreated control animals During the observation period all of the few epithelial buds in the colchicine-treated bursae were populated by lymphoid cells to form mature, histologically normal-appearing lymphoid follicles The number of lymphoid follicles on week 10 how ever, was only about 3 % of that found in the bursal sections of the untreated control animals

The repopulation of each single epithelial bud by lymphoid cells seemed to take place independently because lymphoid follicles were found in different stages of repopulation This repopulation resembled either embryonal development of the bursal follicles (1, 2) or states found in the bursae of the cyclophosphamide-treated chickens spontaneously (5, 7) or following transplantation with bursal lymphoid cells (14–18) The origin of the repopulating lymphoid cells remains unclear, (1) they can be descendants of lymphoid cells which survived the initial necrosis caused by colchicine, or (2) they can be peripheralized bursal lymphocytes, which have returned to bursa or (3) they are lymphocytes from a non-bursal site of B cell development (8)

In the colchicine model no »acute« direct effects on the spleen or caecal tonsils has been demonstrated (12) However, later, the spleen weight, the amount of lymphoid cells in the periellipsoid area and the number of germinal centers in the spleen as

secondary due to total depletion of the lymphoid cells in the bursa On week 10, increased weights and normalized morphology of the spleens indicated their partial recovery This injury-recovery cyclus in the bursa, also partially reflected in the spleen in our present model bears some resemblance to that caused by intramuscular testosterone propionate injection (4)

The chickens which received intraperitoneal injections of cyclophosphamide did not gain as much weight as those treated with colchicine, a fact apparently indicating a more serious systemic effect Besides the total loss of lymphoid cells, no other apparent damage was observed histologically in the bursae of the cyclophosphamide treated chickens Cyclophosphamide did not destroy epithelial buds As reported in earlier studies (6, 7) in cyclophosphamide treated chickens, only a part of numerous epithelial buds are spontaneously populated with lymphoid cells on week 10, while in the present study, all of the few buds in the colchicine group were populated Thus, the cyclophosphamide and the colchicine models also differ in their type of spontaneous lymphoid repopulation of the bursa which is less active in the former The effects of cyclophosphamide on the spleen were evident

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CORRESPONDENCE

HISTOLOGIC TYPING OF BREAST CANCER

Sir –In a recent monograph (9) and in a brief report (8) which appeared in this Journal *Linell et al* proposed a new classification for breast carcinoma Their system was elaborated upon on the observation of a large number of mastectomies in which they recorded a stepwise evolution from »radial scars« into carcinomas they called »tubular«

In our opinion an obvious merit of their extensive investigation has been to intend to apply to breast pathology the acception which appears now established for lung carcinoma (Roessle (11)) that cancer seems to arise in scar tissue They also tried to introduce a concept of dynamic evolution in the process of degeneration suggesting that both histologic type and grade of a tumour does evolve with time We also have been interested in breast pathology and histologic classification of mammary cancer (17 18) Therefore we would like to comment on some of the basic points of *Linell et al* (8 9) studies

1) When the authors claim that »tubule formation as a higher degree of differentiation and of better prognosis is nowadays an obsolete idea« they are in contradiction with their own statements (–» tubule formation is pure in early carcinoma ‹ i e in well differentiated and –

parameters currently in use (19 20)

2) The high percentage of »tubular carcinomas« which reached 21 % (8 9) is in profound disagreement with literature data of a 10 to 20 fold lower frequency This discrepancy is obviously attributable to different histopathological criteria and definition of tubular carcinoma We (17 18) have already stressed the confusion between true tubular carcinoma and well differentiated infiltrating duct carcinoma or even with other subtypes which has appeared repeatedly (*Savino & Koss* (12) So *Hampert* (6) pointed out that even *Haagensen* misused some terms viz by designating true tubular carcinoma under heading of apocrine carcinoma Few reports (*Eusebi et al* (2) *Fisher et al* (4)) suggested that true tubular and lobular carcinoma might be two distinct variants of secretory carcinoma the former being the most differentiated of both subtypes However most authors (*Lagios et al* (7) *Taxy* (14) *Taylor & Norris* (13) *van Bogaert & Maldague* (17 18)) agree with a ductal origin rather than a lobular one

The most frequent misuse – the confusion with highly differentiated infiltrating duct carcinoma. Almost all the papers on the subject of true tubular carcinoma agree with the following histopathologic criteria well-defined tubular structures with angulated contours usually lined by a single layer of bland appearing cells and prominent

lesion they consider a »radial scar« i e not yet a carcinoma although it may progress into tubular carcinoma. They believe that malignant degeneration of the »radial scar« may evolve into 5 subtypes according to ...

conceive that with time the degree of differentiation may decrease it seems rather confusing to name »tubular carcinoma O« a tumour which by definition is almost totally devoid of tubules

3) Radial scars« are said to be also characterized by ...

observation might be correct one could get the wrong impression that elastosis is one of the diagnostic hallmarks As a matter of fact true tubular carcinoma generally show such changes (*Tobon & Salazar* (15) *Tremblay* (16)) however infiltrating duct and lobular carcinoma (*Azzopardi & Laurini* (1) *Martinez Hernandez et al* (10)) scirrhous carcinoma (*Azzopardi & Laurini* (1)) and benign breast tissue (*Martinez Hernandez et al* (10)) have been shown to exhibit the same deposits Moreover studies on this peculiar elastoid substance emphasize that the deposits are found not only in the periductal stroma but also around the blood vessels The latter topography was not mentioned by *Linell et al* (8 9) Finally *Fisher et al* (3) suggested some association between marked elastica and tumours from patients of older age

Conclusion The authors (9) claimed that a new classification system is warranted only when it offers advantages from both a clinical and prognostic point of view Although we fully agree with their statement their study unfortunately was limited to a 1–3 year follow up From a pathologist s point of view we feel that their system is based on the misuse of a name »tubular carcinoma« which has been consacrated in a very precise sense While the authors do recognize all the criteria of

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BRIEF REPORT

ESTIMATION OF THE VOLUME OF HUMAN ANTRAL MUCOSA BY MEASURING THE ANTRAL LENGTH ALONG THE LESSER AND GREATER CURVATURE

H Overgaard Nielsen

Institute of Pathology and Department of Surgical Gastroenterology Odense University Hospital Odense Denmark

Nielsen H O Estimation of the volume of human antral mucosa by measuring the antral length along the lesser and greater curvature Acta path microbiol scand Sect A 89 417–419 1980

A significant positive correlation was found in 11 antrectomy preparations between the product of antral length along the lesser and greater curvatures and the antral mucosal volume (coefficient of correlation 0 9297) This means that the antral mucosal volume can be estimated by measuring the antral length along the curvatures a procedure possible during operation with the organ in situ

Key words Human antrum curvatures mucosal volume

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The surgical treatment of chronic duodenal ulcer as performed today preserves the antrum Antrectomy is only the treatment of choice in some cases of recurrent ulcer This means that direct determination of the antrum area and/or volume can rarely be obtained in duodenal ulcer patients These parametres can be useful i e for determination of the G cell mass and when the antrum size is to be correlated to other parametres It was therefore considered of interest to report a method for the estimation of antral mucosal volume by means of measurement of the length of the antrum along the lesser and greater curvature

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because of early gastric cancer

After removal the antrum was opened along the greater curvature and placed on a sheet of cork The border be

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stripped a its volume determined following the principles of Archimedes by submersion in water As

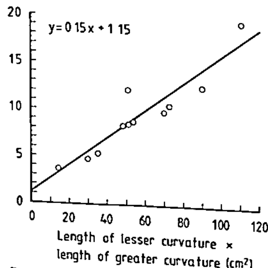


Fig 1 Shows the construction line of regression between antrum mucosa volume and the product of lesser and greater curvature length The line intercept the Y axis of point 1 15 (ml) and have a slope of 0 15 (ml/cm²)

such lesion in the »radial scar« which they apparently consider as a possible precancerous lesion they use the term to designate a wide range of carcinomas with a progressive loss of tubules We can hardly agree with their new classification

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Experimental Pathology
& Cytology LOUIS JACQUES VAN BOGAERT
Unit of the Louvain University School of
Medicine Brussels Belgium

Sir – We thank Dr *van Bogaert* for the interest he has shown to our papers and would like to comment on some of his views

We are glad that Dr *van Bogaert* seems to accept the possibility that tubular carcinomas start in radial scars

1 Dr *van Bogaert* means that we are in contradiction with our own statements about differentiation There is no contradiction if one cites also the following sentence »When the tumours grow larger the progression towards less differentiated structures takes over and the tubules are there only as remnants of the earlier structure of the tumour To us differentiation means the development of higher differentiated structures in an undifferentiated tumour and progression the development of less differentiated elements in a highly differentiated tumour

2 »The high percentage of tubular carcinomas is in profound disagreement with literature data Not at all As we point out on p 63 our material contains a big proportion of tumours found at mass screening The same has been found by other authors cited by us

Dr *van Bogaert* finds it confusing to use the name »tubular carcinoma 0« for a tumour devoid of tubules We cannot find it confusing when reading our text

3 About elastosis »One could get the wrong impression that elastosis is one of the diagnostic hallmarks Naturally we mean this is true and the finding of elastosis in all our tubulo ductal carcinomas is one of our histologic arguments for the progression we describe But elastosis is not pathognomic as stressed on p 122 where we point out that infiltrating lobular

add that it is impossible in this publication to have a long follow up when we use a *modern* material with many small tumours found at mass screening We cannot agree that we misuse the name »tubular carcinoma To us it is not consecrated but subjected to the changing pattern of new knowledge

To the last sentence »We can hardly agree with their new classification« we can only say that several correspondents who have gone through their own materials »with new eyes have agreed with our views

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BRIEF REPORT

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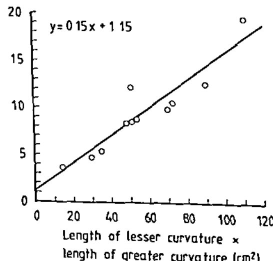


Fig 1 Shows the construction line of regression between antrum mucosa volume and the product of lesser and greater curvature length The line intercept the Y axis of point 1 15 (ml) and have a slope of 0 15 (ml/cm²)

TABLE 1 *Diagnosis Length of Antrum along Lesser and Greater Curvatures and Mucosal Volume in 11 Antrectomy Preparations*

Diagnosis	Antral length lesser curvature (cm) A	Antral length greater curvature (cm) B	Product lesser and greater curvature (A × B) (cm2) C	Antral mucosal volume uncorrected (ml) D	Submucosal volume (% of uncorrected antral volume) E	Antral mucosal volume corrected (ml) (D–E) F
Recurrent duodenal ulcer	6 0	9 0	54 0	10 5	15 50	8 87
Recurrent duodenal ulcer	6 0	8 5	51 0	14 2	15 02	12 07
Prepyloric ulcer	6 5	7 75	50 4	14 0	38 50	8 61
Prepyloric ulcer	4 0	3 5	14 0	3 9	6 67	3 64
Prepyloric ulcer	6 0	5 0	30 0	5 0	5 00	4 75
Gastric ulcer	5 0	7 0	35 0	6 5	15 20	5 51
Gastric ulcer	10 0	11 0	110 0	28 0	31 10	19 29
Gastric ulcer	6 0	8 0	48 0	10 5	18 95	8 51
Gastric ulcer	8 0	9 0	72 0	11 5	8 70	10 50
Gastric ulcer	9 0	10 0	90 0	13 1	5 80	12 30
Early gastric cancer	7 0	10 0	70 0	11 0	10 00	9 90

the stripped mucosa also includes submucosal tissue the proportion of mucosal and submucosal tissue was determined from three 5 μm thick tissue blocks taken from the stripped mucosa, using the point counting technique (1)

The results are seen in Table 1 A significant positive correlation ($P < 0.001$) with a coefficient of correlation of 0 9297 was found between the product of the antral length along the lesser and greater curvatures and the corrected antral mucosal volume The linear equation and construction line of regression between these two variables are shown in Fig 1

Measurement of the antral length can be performed during operation with the stomach in situ using either a pH electrode to establish the border between the pyloric and fundic mucosae or the border can be estimated by microscopic examination of step wise biopsies taken from the pyloric ring and orally along the lesser and greater curvatures

References Nielsen H O Halken S & Lorentzen M Acta path microbiol scand Sect A *88* 255–261 1980

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